

MAPPING AND CHARACTERIZATION OF LOCI CONDITIONING
QUANTITATIVE DISEASE RESISTANCE IN MAIZE

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MAPPING AND CHARACTERIZATION OF LOCI CONDITIONING QUANTITATIVE DISEASE RESISTANCE IN MAIZE

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A range of approaches for QTL analysis was used to identify, characterize and dissect loci conditioning quantitative disease resistance (disease QTL) in maize. By investigating the introgression lines derived from B73 x Tx303, several QTL for resistance to northern leaf blight (NLB) were mapped. Two QTL, Tx303 allele at bin 1.06 (designated *qNLB1.06_{Tx303}*) and B73 allele at bin 1.02 (designated *qNLB1.02_{B73}*), were characterized in derived near-isogenic lines (NILs) using a series of macroscopic and microscopic disease components targeting different stages of NLB development pathogenesis. *qNLB1.06_{Tx303}* was found to be effective mainly against fungal penetration, and *qNLB1.02_{B73}* was effective for reducing the induction of defensive materials surrounding the infection sites, as well as inhibiting hyphal growth into the vascular bundle. Heterogeneous inbred family (HIF) analysis was explored for targeted QTL mapping and NIL development. Two tropical lines, CML52 and DK888, were chosen as donors of alleles based on their superior resistance to multiple diseases. Starting with near-inbred lines derived from B73 x CML52 and S11 x DK888, 73 SSR markers covering 39 bins were used to generate a series of NIL pairs contrasting for chromosomal regions associated with clusters of previously identified QTL for different diseases. By systemically characterizing the NIL pairs for resistance to eight diverse diseases, four disease-specific QTL, a QTL for resistance to NLB and Stewart's wilt, and a QTL for resistance to NLB and anthracnose stalk rot,

were identified. QTL discovered using CML52 HIFs largely conformed to the QTL mapped in a population of recombinant inbred lines (RILs) from the cross of B73 x CML52. A large-effect NLB-specific QTL at bin 8.06 (designated *qNLB8.06_{DK888}*) was characterized for race specificity and allelism with two known major genes in the same region. *qNLB8.06_{DK888}* conferred race-specific resistance, and is identical, allelic, or closely linked and functionally related to *Ht2*. Using high-resolution breakpoint analysis, *qNLB8.06_{DK888}* was delimited to a region of ~0.46 Mb spanning from 143.92-144.38 Mb on the B73 physical map. Out of 12 annotated genes in the region, three candidate genes including two encoding protein kinases and one encoding a protein phosphatase were identified.

BIOGRAPHICAL SKETCH

The author was born and raised in Kaohsiung, a city located in southern Taiwan. The author's long-time interest in life science led her to study in Department of Plant Pathology and Entomology at National Taiwan University, where she earned both bachelor and master degrees. She learned about science and research, by working with mycoparasitic fungi, under the guidance of Dr. Shean-Shong Tzean. She later worked in the Bureau of Animal and Plant Health Inspection and Quarantine in Taiwan for four years, taking charge of the coordination of plant pest detection, monitoring, risk assessment, and international cooperation. The experience of working in the regulatory system broadened her view of domestic and international issues relating to plant protection and agriculture. With attempt to become a more professional plant pathologist, she started her doctoral study at Cornell in 2004. The author's major scientific interest is in exploring the genetics underlying the agriculturally-important quantitative disease resistance.

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CHAPTER 1

RESISTANCE LOCI AFFECTING DISTINCT STAGES OF FUNGAL PATHOGENESIS IN MAIZE: USE OF INTROGRESSION LINES FOR QTL MAPPING AND CHARACTERIZATION¹

ABSTRACT

To test a series of hypotheses about the nature of quantitative disease resistance, a set of introgression lines of maize was used for mapping and characterization of quantitative trait loci (QTL) conditioning resistance to a fungal pathogen, *Setosphaeria turcica*, the causal agent of northern leaf blight (NLB). Several introgression lines showing levels of resistance to NLB different from the recurrent parent, B73, were identified among a set of 82 introgression lines. Seven lines were confirmed as more resistant or susceptible than B73 in a subset of 15 selected introgression lines tested at two sites. Two NLB QTL were validated in BC₄F₂ segregating populations and advanced introgression lines. These loci, designated *qNLB1.02*_{B73} (the B73 allele at bin 1.02) and *qNLB1.06*_{Tx303} (the Tx303 allele at bin 1.06), were investigated in detail by evaluating the introgression lines with a series of macroscopic and microscopic disease components targeting different stages of NLB development. Data from repeated greenhouse and field trials revealed that *qNLB1.06*_{Tx303} is effective mainly against fungal penetration, while *qNLB1.02*_{B73} is effective for inducing the accumulation of callose and phenolics surrounding infection sites, as well as inhibiting hyphal growth into the vascular bundle, and the subsequent

¹ Joy Longfellow, Ellie Walsh, and Zura Kurdieh participated in phenotypic data collection in the field and greenhouse trials in New York. George Van Esbroek and Peter Balint-Kurti conducted the field trials in North Carolina.

necrotrophic colonization in the leaves. Both QTL were as effective in both juvenile and adult plants, and *qNLB1.06_{Tx303}* showed greater effectiveness in the field than in the greenhouse. Evaluating the introgression lines for response to a number of important diseases suggested that in addition to NLB, *qNLB1.02_{B73}* conditions resistance to Stewart's wilt and common rust, and *qNLB1.06_{Tx303}* confers resistance to Stewart's wilt. The non-specific resistance may be attributed to the pleiotropic effects of a single gene or the close linkage of different genes conferring disease-specific resistance.

INTRODUCTION

Pathogenesis is a series of events occurring in a host-pathogen interaction, including infection, colonization, reproduction, and dissemination of the pathogen in the host. Genetic variation in both host and pathogen can have quantitative or qualitative effects on the success of the pathogen at each stage. Many plant genetic factors that modulate pathogenesis have been discovered. The best-known type is major resistance genes known as R-genes, which provide high levels of resistance or even complete immunity. R-gene mediated resistance is typically initiated by a “gene-for-gene” interaction; the recognition of a pathogen effector by a host protein encoded by the R-gene, followed by the induction of the hypersensitive response (HR), the production of antimicrobial metabolites such as phytoalexins, and the expression of pathogenesis-related (PR) proteins (Jones and Dangl 2006). This type of interaction, typically resulting in a race-specific but highly effective defense response against pathogenic invasion, is sometimes known as qualitative resistance. Quantitative resistance, on the other hand, typically confers intermediate levels of resistance and is believed to be controlled by a set of plant defense-related genes distinct from, or

partially-overlapping with, those involved in qualitative resistance (Poland et al. 2009).

Although each quantitative resistance locus conditions a relatively small effect on pathogenesis, this type of resistance is of agricultural interest because it is generally more durable; multiple genes with minor effects lead to lower selection pressure and greater complexity to overcome (Parlevliet 2002). Quantitative resistance has thus been incorporated in a range of crop cultivars by plant breeders. A large number of quantitative trait loci (QTL) for disease resistance have been mapped in plants (reviewed by Poland et al. 2009; Young 1996), but little is known about the underlying genetic basis or defense mechanism. A range of genetic mechanisms controlling basal resistance, defense signaling pathways, detoxification, morphology, and development in the plant host, is hypothesized to be associated with reducing disease progress (Poland et al. 2009). A small number of quantitative resistance genes have been cloned recently (Broglie et al. 2006; Fu et al. 2009; Fukuoka et al. 2009; Krattinger et al. 2009; Manosalva et al. 2009), implicating diverse host functions in quantitative resistance.

Given that diverse host functions affect quantitative resistance, it is likely that QTL act at different stages of pathogenesis. The ways in which quantitative resistance affects different stages of pathogenesis has been addressed, to a limited extent, by comparing trait values obtained using distinct (usually macroscopic) disease components. In most (or probably all) of the phytopathosystems analyzed to date, differences in various disease parameters can be observed among plant genotypes. Previous QTL studies for foliar diseases have mapped distinct loci associated with incubation period, lesion number, lesion size, or diseased leaf area, indicating that defense genes affecting lesion formation and lesion expansion may not be the same. In breeding programs, selection for decreased lesion length or lesion numbers can have

insignificant effects on incubation period or disease severity. These observations suggest that distinct resistance mechanisms govern macroscopic components of resistance.

More insights into the role of a given disease QTL in limiting pathogenesis can be gained through histopathological analysis. While biochemical and microscopic functional analyses have been applied to investigate major gene resistance and fungal pathogenicity factors [reviewed by Vidhyasekaran (2007)], few studies have reported the effect of individual QTL on distinct stages of pathogenesis from a microscopic view [exceptions include (Moldenhauer et al. 2008)]. If QTL effective at specific stages of pathogenesis can be identified, combining favorable alleles for complementary QTL (eg. for infection and colonization) will likely provide greater levels of resistance.

Northern leaf blight (NLB; also known as turcicum blight) of maize was used as a model system to identify and characterize disease QTL at the macroscopic and microscopic levels. NLB, caused by *Setosphaeria turcica* (anamorph *Exserohilum turcicum*, syn. *Helminthosporium turcicum*), is one of the most prevalent foliar diseases in most maize-growing regions of the world. The disease causes periodic epidemics associated with significant yield losses (Perkins and Pedersen 1987; Pingali and Pandey 2001; Raymundo and Hooker 1981; Ullstrup and Miles 1957), particularly under conditions of moderate temperature and high humidity (Carson 1999).

Qualitative and quantitative forms of resistance against *S. turcica* are available in maize germplasm (Welz and Geiger 2000; Wisser et al. 2006), and have been widely utilized alone or in combination in resistance breeding programs (Pratt and Gordon 2006). A few histological studies have revealed the pathogenesis of *S. turcica* on maize leaves by staining, whole mount and serial dissection (Hilu and Hooker 1964, 1965; Jennings and Ullstrup 1957; Knox-Davies 1974). Marked phenotypic variation

in symptom development has been observed among diverse maize lines in our multiple field and greenhouse trials. How macroscopic and microscopic phenotypes relate to specific QTL remains to be determined.

To answer questions concerning individual QTL effects, such as testing the hypothesis that distinct QTL act at different stages of pathogenesis, well-defined genetic stocks that differ only at specific loci are required. For maize, a set of introgression lines named TBBC3 is currently available (Szalma et al. 2007). A population of introgression lines is composed of introgression lines carrying one or a few chromosomal segments of a donor genotype in the genetic background of a recurrent parent. Introgression lines have been successfully used to study QTL in maize (Stuber et al. 1999; Szalma et al. 2007), rice (Li et al. 2005), barley (Brown et al. 1988; Toojinda et al. 1998), tomato (Eshed and Zamir 1995), and Arabidopsis (Keurentjes et al. 2007). While QTL analysis using recombinant inbred lines (RILs) provides greater statistical power in detecting QTL (Kaepler 1997), RIL-based approaches have limitations in estimating QTL effects (Eshed and Zamir 1995; Keurentjes et al. 2007; Remington and Purugganan 2003). Introgression lines can be efficiently used to produce near-isogenic lines (NILs), which permit careful analysis of phenotypic effects associated with introgressed segments (Eshed and Zamir 1995; Szalma et al. 2007).

NILs allow many long-standing questions about quantitative disease resistance to be addressed, such as the relationship between disease QTL and plant maturity, the interaction of QTL and environmental factors, and the specificity of resistance conditioned by QTL. The interplay between disease resistance and plant development has been widely recognized (Whalen 2005) yet remains poorly understood. In general, the resistance in adult plants or older leaves is greater than in juvenile plants or younger leaves [eg. Kim *et al.* (1987), Century *et al.* (1999), Kus *et al.* (2002)], and a

correlation between resistance and flowering time has been found (Collins et al. 1999; R. Wissner, J. Kolkman, and P. Balint-Kurti, pers. comm.). Some QTL effects may thus be specific to certain plant developmental stages. In addition, the expression and effectiveness of many genes/QTL have been observed to be regulated by environmental conditions. Another issue of fundamental and practical interest is whether a disease QTL confers specific or broad-spectrum resistance. A single locus can condition resistance to more than one disease, if it encompasses linked QTL effective against different diseases, or its underlying genes are involved in broad-spectrum resistance pathways.

Here, we describe the use of introgression lines/NILs-based analysis for QTL mapping and macro-/microscopic characterization in the maize – *S. turcica* pathosystem. We used a population of introgression lines with each line carrying a few chromosomal segments from the moderately resistant maize genotype Tx303 in a homogeneous genetic background of B73 to map QTL for resistance to NLB. To better understand the nature of quantitative resistance, we assembled a panel of conventional and novel disease components targeting different stages of disease development. These were used to demonstrate that two distinct QTL affect different stages of pathogenesis. The QTL were further characterized to shed light on three secondary hypotheses: (1) that disease QTL differ by host developmental stage (young versus adult), that (2) their performance changes across environments (field versus greenhouse), and (3) that they condition broad-spectrum resistance. Overall, our study provides the knowledge on disease QTL that will facilitate more effective and efficient application of quantitative resistance in crop protection.

MATERIALS AND METHODS

1. Plant materials

A set of 82 TBBC3 introgression lines was provided by J. Holland of the USDA-ARS unit at North Carolina State University. The TBBC3 (for Tx303 by B73 Backcross 3) population was the most extensively developed set of introgression lines available at the time for public use in maize. It was originally developed by C. Stuber at North Carolina State University (Stuber et al. 1999). The population was derived from an initial cross of Tx303 and B73, followed by backcrossing to B73 for three generations. Each line was then selfed for several generations to attain homozygosity. Genotypic information was publicly available for each line, consisting of 14 restriction fragment length polymorphism (RFLP) and 116 simple sequence repeat (SSR) markers across the genome. Each line was known to carry one or more Tx303 introgressions, covering on average 5% of the genome, in the background of the sequenced reference maize line B73. Taken together, the set of introgression lines represents ~89% of Tx303 genome (Szalma et al. 2007).

To validate and characterize the effects of Tx303 introgressions, several BC₄F₂ populations were developed by crossing selected TBBC3 lines to B73. Sets of BC₄F₃ and BC₄F₄ lines carrying different introgression(s) were subsequently derived by single-seed descent. After four generations of marker-assisted backcrossing, the BC₄F₃ and BC₄F₄ lines were designated as NILs.

2. Assessments of northern leaf blight

Pathogen strains. Experiments carried out in New York were inoculated with *S. turcica* isolate NY001. This isolate has the race 1 phenotype based on race identification trials (details in Chapter 3, Table 3.1). That is, under the standard

greenhouse conditions established for NLB assays (Leonard et al. 1989), NY001 is compatible on the test maize line carrying the *Ht1* gene for resistance and is incompatible on lines carrying *Ht2* and *Ht3* and *Htn1*. Experiments at Clayton NC were conducted with a mixture of isolates representing race 1, race 23, and race 23N of *S. turcica* as the inoculum.

Inoculum preparation. For preparation of liquid inoculum, *S. turcica* was cultured for two to three weeks on lactose – casein hydrolysate agar (LCA) plates under a 12 hr/ 12 hr normal light-dark cycle at room temperature. The conidia were then dislodged from the plates with sterile ddH₂O and a glass rod. The spore suspension was filtered through four layers of cheesecloth, and adjusted to 4×10^3 conidia per ml (0.02% Tween 20) with the aid of a haemocytometer. Solid inoculum was prepared by culturing *S. turcica* on sorghum grains for two to three weeks under a 12 hr/12 hr normal light-dark cycle at room temperature in plastic milk jugs. For each jug, 900 ml of sorghum grains were soaked overnight in 600 ml of water in a milk jug covered with a ventilated lid. The jug was then autoclaved twice at 121 °C, 15 lb/cm², for 25 minutes per run. The jugs were inoculated by dividing the spore suspension produced from one heavily colonized LCA plate among five jugs. Jugs were shaken every day until use to prevent caking and accelerate fungal colonization.

Plants at the six-leaf stage were used for inoculation. The inoculation technique utilized depended on the specific objectives of the experiment. In the field trials in NY, plants were inoculated with both liquid (0.5 ml of spore suspension) and solid inoculum (1/4 teaspoon, ~1.25 ml of colonized sorghum grains) placed in the whorl. This was done to ensure the viability of inoculum across a range of conditions (under optimal conditions, the liquid inoculum was considered most effective, while the solid inoculum was considered to perform more effectively under dry conditions). In the field trials in NC, *S. turcica* was cultured as described above. The inoculation was

conducted by placing ~20 grains of sorghum colonized with *S. turcica* in the whorl, as described by (Carson 1998).

In greenhouse trials, whorl inoculation was carried out for assessing incubation period and lesion expansion in preliminary tests on inbred lines, and on individual plants in the segregating populations. Plants were inoculated with 0.5 ml of spore suspension (4×10^3 conidia per ml, 0.02% Tween 20). Spraying inoculation was performed for detailed QTL characterization, as it provides significantly better differentiation for NLB evaluation (data not shown). The spraying method is especially preferable for microscopic examination and real-time PCR quantification. A higher number of spores can be evenly distributed on leaf surface with spraying, making the subsequent sampling more effective and accurate. About 0.5 ml of concentrated spore suspension (5×10^4 conidia per ml, 0.02% Tween 20) was evenly sprayed on the first fully expanded leaf with an airbrush (Badger® Model 150) at 20 psi. After inoculation, the plants were kept overnight in a mist chamber at > 85% RH, then maintained at 22 °C day/ 18 °C night temperature with a 14 hr-light/ 10 hr-dark cycle.

3. Phenotypic characterization of resistance to NLB

Field experiments were conducted at Cornell's Robert Musgrave Research Farm in Aurora, NY and Central Crops Field Station in Clayton, NC. Plants were evaluated for different disease parameters and for days to anthesis (DTA). DTA, which was only assessed for field-grown plants, was scored on a row basis when > 50% of the plants in a row started to shed pollen. An overview of various disease components used in this study and their corresponding stages during NLB development is summarized in Table 1.1. The evaluation method for each parameter is illustrated as below.

Microscopic analysis. Two microscopy techniques were applied to investigate differential development of *S. turcica* in the near-isogenic lines: trypan blue staining

Table 1.1 Overview of disease components used to target different stages of northern leaf blight (NLB) development.

Disease component	Description (unit)	Targeted disease development stage (s)	Evaluation	Literature
Incidence of multiple appressoria ^a	The incidence of > 1 appressorium developed from each germinated conidium (%)	Pre-penetration	Trypan blue staining and microscopy	Knox-Davies 1974; Vélez 2005
Infection efficiency ^a	The incidence of successful infection per germinated conidium (%)	Penetration into the epidermal cell	Trypan blue staining and microscopy, KOH-aniline blue fluorescence microscopy	Knox-Davies 1974; Vélez 2005
Accumulation of callose and phenolics ^a	Diameter of enhanced fluorescing area surrounding the infection site (µm)	Intercellular and intracellular hyphal growth from primary infected cell to surrounding mesophyll cells	KOH-aniline blue fluorescence microscopy	Hood and Shew 1996
Vascular invasion efficiency ^a	The incidence of hyphae entering vascular bundles per infection site (%)	Hyphal growth into the vasculature	KOH-aniline blue fluorescence microscopy	Hood and Shew 1996
Fungal biomass ratio ^a	The percentage of fungal DNA divided by the total DNA in the infected leaf tissues (%)	Overall fungal growth in leaves before the appearance of necrotic lesions	DNA-based real-time quantitative PCR	Qi and Yang 2002
Incubation period (IP)	The number of days from inoculation to the appearance of the first lesion on a plant (days)	Xylem plugging due to extensive hyphal growth in the vascular veins	Visual examination	Brewster et al. 1992; Carson 1995; Carson and Van Dyke 1994; Schechert et al. 1999; Welz et al. 1999a; Welz et al. 1999b
Lesion expansion (LE) ^a	The longitudinal expansion of a lesion per day (mm)	Destructive hyphal growth in primary inoculated leaves	Digital caliper measurement	Brewster et al. 1992; Sigulas et al. 1988

Table 1.1 (Continued)

Disease component	Description (unit)	Targeted disease development stage (s)	Evaluation	Literature
Diseased leaf area (DLA)	The percentage of infected leaf area of the entire plant, disregarding decayed bottom leaves (%)	Destructive hyphal growth on the leaves of a entire plant, caused by primary and secondary inoculum	Visual examination	Brown et al. 2001; Freymark et al. 1994; Schechert et al. 1999; Welz et al. 1999a; Welz et al. 1999b
Disease severity ^b	The severity of infected leaf area of the entire plant (scale 1-10, 1: little diseased area)	Destructive hyphal growth on the leaves of a entire plant, caused by primary and secondary inoculum	Visual examination	Balint-Kurti et al. 2007
Area under the disease progress curve (AUDPC)	Total area under the graph of DLA or disease severity (area unit)	An overall destructive hyphal growth on a plant throughout the season	Calculated from visual examination scores	Schechert et al. 1999; Welz et al. 1999a

^a First time of being used as a disease component for evaluating NLB resistance.

^b Only applied in the 2006 trial in North Carolina.

and KOH - aniline blue staining. In greenhouse trials, infected leaf samples were harvested from individual plants. In the field trial, for the purpose of obtaining a sufficient number infection sites for examination, samples (per genotype per block) were collected from four plants in a row and pooled for subsequent treatments.

Trypan blue staining was performed as previously described (Knox-Davies 1974; Vélez 2005) with some modifications for analyzing fungal penetration. Infected tissues were collected at two days post inoculation (dpi) from plants in the greenhouse, and at 3 dpi from plants in the field trials. Leaf samples were cut into $1 \times 1 \text{ cm}^2$ segments, cleared first in an acetic acid: ethanol (1:3, v/v) solution overnight, then in an acetic acid: ethanol: glycerol (1:5:1, v/v/v) solution for at least 3 hours. The samples were subsequently incubated overnight in a staining solution of 0.01% (w/v) trypan blue in lactophenol, and rinsed then stored in 60% glycerol until examination. Specimens were transferred onto microscopic slides and examined under a compound microscope. Fifty to 60 germinated conidia were assessed per individual plant (greenhouse) or per row (field).

A modified KOH-aniline blue fluorescence technique (Hood and Shew 1996) was used to visualize the growth of fungal hyphae inside the infected leaves and the accumulation of (plant-produced) callose and phenotypic compounds around the infection sites. Infected leaves were sampled at 4 dpi and 7 dpi in greenhouse trials, and at 6 dpi in the field trial. The samples were cut into $1 \times 1 \text{ cm}^2$ segments, incubated in 1M KOH at room temperature for 24 hours, then autoclaved at 121°C , 15 lb/cm^2 for 2-5 min. Autoclaving time was adjusted according to the rigidity of leaves, which varied with plant genotype and maturity. The autoclaved specimens were rinsed in ddH₂O three times, then stored in autoclaved ddH₂O until examination. Specimens were carefully placed on microscopic slides and mounted in a staining solution of 0.05% aniline blue in 0.067 M K₂HPO₄ (prepared at least 2 hrs prior to use). Thirty

five to 40 germinated conidia were checked per individual plant (greenhouse) or per row (field) under a Zeiss fluorescence microscope with a G365 excitation filter, a FT395 dichromatic beam splitter, and an LP420 barrier filter.

Quantitative real-time PCR for quantifying fungal colonization. DNA-based real-time PCR was performed as described by Qi and Yang (Qi and Yang 2002) with some modifications. The specific pair of primers for *S. turcica*: forward: 5'-TCTTTTGCGCACTTGTTGTT and reverse: 5'-CGATGCCAGAACCAAGAGAT, were designed based on the internal transcribed spacer 1 (ITS1) of ribosomal DNA gene in *S. turcica*. The ITS1 sequence (GenBank: AF163067.1) was obtained from the nucleotide database of the National Center for Biotechnology Information (NCBI). PCR amplification resulted in a specific fragment of 170 base pairs.

Inoculations experiments were performed three times in the greenhouse. Five plants of B73, Tx303, and the two NILs were spray-inoculated. The same amounts of infected tissue (0.12 g per plant) were collected at 9 dpi from the middle part of each leaf. Leaf samples were ground with liquid nitrogen and DNA was extracted following the protocol described above. The extracted DNA from each individual plant was dissolved in 100 µl TE buffer. Total DNA concentration was determined using the PicoGreen® dsDNA quantitation assay kit (Invitrogen, Eugene, Oregon, USA). Fungal DNA was quantified using real-time PCR. The ratio of fungal biomass in maize leaves was computed from the amount of fungal DNA divided by total DNA.

Each real-time PCR reaction was performed in a total volume of 25 µl, containing 12.5 µl of iTaq SYBR® Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA, USA), 3 µl of 7.5-fold diluted DNA from an infected plant and 300 nM of each forward and reverse primer. PCR samples were incubated in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with thermal cycling parameters of 95 °C for 2 min followed by 40 cycles of 15 sec at 95

°C and 30 sec at 56 °C. Two standard curves were constructed by mixing a series of *S. turcica* DNA (0, 1, 10, 50, 100, 500 and 1000 pg) with 50 ng of maize DNA extracted from non-inoculated B73 and Tx303 plants, respectively (Fig. 1.6A). The quantification of fungal biomass in infected B73 and derived NILs was based on the standard curve created from mixing with a constant amount of B73 DNA, while the quantification for DNA from infected Tx303 was based on the second standard curve. Three technical replicates were carried out in individual plates for both Picogreen quantification and iTaq SYBR Green PCR, with the samples for standard curves repeated twice in the same plates.

Incubation period (IP). Individual plants were checked every day after 7 dpi for the appearance of the first wilted lesions. The number of dpi when the first lesions were observed was scored as the IP. In the trial at Aurora NY, IP scores were rated for individual plants, then averaged for the rows. In the trial at Clayton NC, IP was recorded on a row basis when > 50% of the plants in a row started showing lesions.

Lesion expansion (LE). Around two to three weeks after inoculation, three lesions per plant were randomly chosen for measurement. Lesion margins were marked and then measured 10-14 days later for the longitudinal extension with a digital caliper. The expansion measurements taken from three lesions were averaged, and divided by the number of days from the marking until measurement of the lesions. The LE of an individual plant was denoted as “the average change in length per day”, and the LE score for a row was the mean of LE values taken from all the plants in the row.

Primary diseased leaf area (PrimDLA). Primary DLA was rated as the percentage of infected leaf area of the inoculated leaves in the 2006 trial in NY. It was scored once on a row basis at around three to four weeks after inoculation.

Diseased leaf area (DLA). DLA was rated as the percentage of infected leaf area of the entire plant, disregarding decayed bottom leaves. DLA was rated on a row basis for fixed lines, and on individual plants for testing trait-marker association in segregating populations. DLA was rated three to four times per season, at an interval of 10-14 days. The first DLA was scored at one to two weeks after observing the onset of secondary infection.

Disease severity. Disease severity was rated on a row basis four times through the season in the 2006 trial in NC. The severity score was based on a 1 to 9 scale corresponding to the percentage of infected leaf area on primarily the ear leaf as well as the leaves above and below the ear leaf (severity 1: 0%, 2: 12.5%, 3: 25%, ..., 9: 100%).

Area under the disease progress curve (AUDPC). The AUDPC was calculated as $\sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})(t_{i+1} - t_i)}{2}$, where y_i = DLA or disease severity at time i , $t_{i+1} - t_i$ = day interval between two ratings, n = number of ratings (Gaurilcikiene et al. 2006).

4. Evaluation for multiple disease resistance

Stewart's wilt. *Pantoea stewartii* (syn. *Erwinia stewartii*) strain PsNY003, originally collected in NY in 1991 from naturally infected leaves of sweet corn, was obtained from Helene Dillard of Cornell University. Inoculum was prepared by streaking bacteria from 50% glycerol stock (preserved at -80 °C) on nutrient agar plates. After incubating at room temperature under a 12 hr/12 hr normal light-dark cycle for two days, the bacteria in each petri dish were transferred to 200 ml of nutrient broth and shaken overnight at 110 rpm at room temperature. Cultures from different flasks were pooled and quantified using a haemocytometer. For inoculation,

bacterial cells were adjusted to a final concentration of 10^7 colony forming units (cfu) /ml with sterilized 0.1 M NaCl solution (Suparyono and Pataky 1989). Plants at five to six-leaf stage were inoculated with *P. stewartii* following the pinprick method (Blanco et al. 1977; Chang et al. 1977) with some modification. Multiple-pin inoculators was made with 30 T-pins (1.5 inch long), pieces of 5.5 cm x 6.5 cm sponge and cork board (3/8 inch thick) fastened on two arms of a tong with rubber bands. Each plant was inoculated by piercing the whorl leaves twice with the inoculator pre-dipped in bacterial suspension. Primary diseased leaf area was rated as the percentage of infected area of the inoculated leaves in the 2008 trial in NY. It was scored twice (two and three weeks after inoculation) on a row basis and the scores were averaged.

Anthracnose stalk rot. A New York isolate of *Colletotrichum graminicola* (teleomorph: *Glomerella graminicola*) (isolate Cg151) was obtained from G. Bergstrom of Cornell University. Inoculum was prepared as describe by (Muimba-Kankolongo and Bergstrom 1990). Each plant was inoculated with 1 ml of 10^6 conidia per ml (0.02% Tween 20), introduced into the stalk, when more than 50% of the plants in every row were tasseling (Keller and Bergstrom 1988). The first elongated internode above the brace roots was punctured with an ice pick, and a 1 ml pipette tip was immediately inserted into the internode. One ml of spore suspension was then dispensed into each tip. The pipette tip was removed after the inoculum was absorbed by the plant. Four weeks after inoculation, stalks were split longitudinally and the percentages of discolored area of individual internodes were visually rated (Keller and Bergstrom 1988) and summed for analysis. In 2007, eight consecutive internodes were scored from four plants per row (inoculation was conducted in NLB plot); in 2008, six consecutive internodes were scored from eight plants per row.

Common smut. In the 2007 trial, plants in NLB plots were evaluated for the development of ear galls and stalk galls resulting from natural infection. Artificial

inoculation was conducted in the 2008 trial using six compatible strains of *Ustilago maydis* (UmNY001, UmNY002, UmNY003, UmNY004, UmNY008 and UmNY009), which were isolated from naturally infected smut galls collected at Aurora NY in 2007. The isolation was conducted following Thakur *et al.* (1989), and the compatibility of sporidial isolates was determined by mating tests on potato-dextrose agar (PDA) plates (Puhalla 1968). An inoculation method described previously (du Toit and Pataky 1999) was used with modification. Inoculum was prepared by first culturing the compatible strains in 10 ml of potato-dextrose broth (PDB) in separate 15 ml falcon centrifuge tubes on a shaker at 100 rpm at room temperature for 1 day. Flasks of 150 ml PDB were seeded with sporidial suspension from centrifuge tubes, then incubated overnight on a shaker at 100 rpm at room temperature. The suspension was adjusted to a final concentration of 10^6 sporidia per ml (0.02% Tween 20) with sterilized distilled water and the aid of a haemocytometer. Equal amounts of sporidia from the six compatible strains were mixed prior to inoculation. The inoculation was conducted by injecting 2 ml of mixed sporidial suspension in the first ear of each plant. Non-pollinated (shoot-bagged) ears were injected at the time that the silks of most ears had emerged 1-5 cm. Every plant in the row was rated from four weeks after inoculation for ear galls on a 0-10 scale, corresponding to the number and size of galls and the disease severity of the entire plant (0 = no smut galls and 10 = a dead smut-infected plant). Stalk galls resulted from natural infection were also scored in the 2008 trial on the same scale.

Common rust. Urediniospores of *Puccinia sorghi* were collected from naturally infected leaves at Aurora NY in 2007. Inoculum was increased on three to four-leaf stage seedlings of susceptible sweet corn in the greenhouse. About 300 mg of stock urediniospores (preserved at -80 °C) were suspended in 100 ml of Sortrol oil (Chevron Phillips Chemical Company, Phillips, TX, USA) (Webb et al. 2002) and evenly

applied on leaves with a spray gun (Preval, Yonkers, NY, USA). Plants were kept overnight in a mist chamber at > 85% RH, then grown for two more weeks until *Puccinia sorghi* sporulated vigorously. The urediniospores were collected by agitating infected leaves with matured rust pustules in distilled water and filtering through four layers of cheesecloth. Spore suspension was adjusted to a final concentration of 2×10^5 urediniospores per ml with the aid of a haemocytometer. Field plants were inoculated at six to eight-leaf stage by adding 1 ml of spore suspension (0.02% Tween 20) in the whorl (Pataky and Campana 2007). Evaluation for disease severity was based on a 0-10 scale with 0.5 increments, corresponding to the percentage of infected leaf area of the entire plant (0 = no disease, 1 = 10%, ..., 10 = 100%). Disease severity was scored three times at 9-day intervals from four weeks after inoculation in 2008, and at 15 to 20 day-intervals from 10 days after inoculation in 2009. AUDPC was calculated from the three severity scores as described above.

5. DNA extraction and genotyping

Plant genomic DNA was extracted following a mini-prep CTAB extraction protocol modified from the methods of Doyle and Doyle (1987) and Qiu *et al.* (2006). About 0.1 g of fresh or lyophilized leaf tissue and a stainless steel ball (5/32 inch diameter, OPS Diagnostics, NJ, USA) were loaded in each well of a 96-well plate (Corning® Costar 96 Well Polypropylene Cluster Tubes). The plate was kept at -80°C for at least overnight, then placed in Genogrinder 2000 (SPEX CertiPrep Inc., Metuchen, NJ, USA) to pulverize the frozen tissues at 450 strokes/min for 50-120 sec. In each tube, the powder was dispersed in 500 µl of CTAB extraction buffer [2 % (w/v) hexadecyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.2% (v/v) of 2-mercaptoethanol; 2-mercaptoethanol was added prior to use], and incubated at 65 °C for 30-50 min with occasionally

inversion. Chlorophorm/isoamyl alcohol (400 µl of 24:1, v/v) was added and mixed thoroughly with CTAB buffer by inversion. After centrifuging at 5200 rpm for 15 min at room temperature, the aqueous phase (supernatant) was transferred to a new plate, and 300 µl of cold isopropanol was added. The plate was gently inverted for several times then incubated at -20°C overnight. The precipitated DNA was recovered by 12 min of centrifugation at 5200 rpm at 4 °C. The supernatant was discarded, and the DNA pellet was desalted by rinsing with 300 µl of 70 % then 100% ethanol. The air-dried pellet was dissolved in 100-150 µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). To extract fungal DNA, *S. turcica* mycelium was scraped from the culture plate with a spatula, and ground in liquid nitrogen with a pestle and mortar. The ground mycelium was transferred to a 1.5 ml eppendorf tube for mini-prep CTAB extraction following the steps as described above. Simple sequence repeat (SSR) markers were used for genotypic analysis. A single-reaction nested PCR method (Schuelke 2000), which allows incorporation of fluorescent dye in PCR product using the specific primer pair along with a fluorescently-labeled universal primer, was applied with modification. Each PCR reaction was performed in a total volume of 13 µl, containing final concentrations of 1x PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% (v/v) Triton X-100], 1.5 mM MgCl₂, 1 M betaine, 0.16 µM fluorescently-labeled universal M13(-21) primer, 0.04 µM forward-specific primer with M13(-21) tail at its 5' end, 0.16 µM reverse-specific primer, 1-3 units Taq polymerase, and 20-50 ng template DNA. The thermal cycling parameters were: 1 cycle of 94°C (5 min), 30 cycles of 94 °C (30 sec) / 56 °C (45 sec) / 72 °C (45 sec), 8 cycles of 94 °C (30 sec) / 53 °C (45 sec) / 72 °C (45 sec), and the final extension at 72 °C (10 min). Amplicons labeled with different fluorescent dyes were multiplexed (combining up to four PCR reactions), mixed with GeneScan-500 LIZ size standard (Applied Biosystems), and analyzed on the Applied BioSystems 3730xl DNA

Analyzer at Biotechnology Resource Center at Cornell University. A total volume of 10 μ l containing 9 μ l formamide, 0.7 μ l PCR product per specific primer pair, and 0.05-0.1 μ l size standard was used. The sizes of amplicons were scored in GeneMapper v. 3.0 (Applied Biosystems).

6. Experimental design

The full set of 82 TBBC3 lines, the subset of 15 TBBC3 lines, and the derived sets of BC₄F₃ and BC₄F₄ NILs were evaluated in the field following the resolvable incomplete block design (also known as an alpha design; Patterson and Williams 1976). The 82 TBBC3 lines were evaluated at Aurora NY for IP, PrimDLA, DLA, and days to anthesis (DTA). To precisely estimate the effects of Tx303 introgressions, every experimental row was grown next to a row of B73 (B73 in every third row). The whole design had two replicates with 14 blocks per replicate, and six experimental rows plus three B73 rows per block. A parallel experiment was conducted on the 82 TBBC3 lines at Clayton NC with two replicates, nine blocks per replicate and 10 rows per block. The B73 rows were included in every other block for the control (five B73 rows per replication). Disease severity and DTA were evaluated. The field trials on the 15 selected TBBC3 lines were conducted at Aurora NY and Clayton NC following the alpha design with B73 in every third row as described above. The 15 lines were selected from those significantly different from B73 in NLB resistance (based on the first-year results), and/or from the lines carrying Tx303 introgression(s) corresponding to previously identified NLB QTL. In each location, there were four replicates with four blocks per replicate, and four experimental rows plus two B73 rows per block. The IP, DLA, and DTA were evaluated. An additional disease component, lesion expansion (LE), was evaluated at Aurora NY as well. During the same field season, to understand the effectiveness of identified QTL in adult plants, the same 15 selected

TBBC3 lines were grown in a separate field plot at Aurora NY, following identical arrangement and design with three replications. The plants were inoculated two weeks before flowering (rather than the usual six-leaf stage), and evaluated for IP and DLA.

To associate resistance with specific introgressions, several BC₄F₂ populations were genotyped and phenotyped for IP, LE, and DLA in either greenhouse or in the field at Aurora NY. Individual plants of each population were grown within a single block with B73 rows as the border. For QTL confirmation, derived BC₄F₃ and BC₄F₄ NILs were evaluated at Aurora NY for IP, LE, DLA, and DTA, following the previously described alpha design with B73 rows in every third row. The experiment was planted in two replicates, with five blocks per replicate, and four experimental rows plus two B73 rows per block.

To test whether the identified NLB QTL confer resistance to other important maize diseases, derived BC₄F₃ and BC₄F₄ NILs were evaluated at Aurora NY for anthracnose stalk rot (ASR) and common smut in 2007 and 2008, and for Stewart's wilt and common rust in 2008 and 2009. In 2007, the assessments of artificially inoculated ASR and naturally occurring common smut were conducted on the plants in NLB trial. In 2008 and 2009, derived NILs were grown in separate field plots for different disease evaluations. Trials for Stewart's wilt and common rust were planted following the previously described alpha design, with two replicates, five blocks per replicate, and four experimental rows plus two B73 rows per block. Trials for ASR were planted following the same alpha design, with two replicates, two blocks per replicate, and seven experimental rows plus four B73 rows per block. Plants in the trials of common smut were randomized in two replicates.

Four genotypes were used for microscopic analysis and DNA-based real time PCR quantification: B73, Tx303, TBBC3-38-05F (the NIL with *qNLB1.06*_{Tx303}), and TBBC3-42-10E-02 (the NIL with *qNLB1.02*_{Tx303}). Three greenhouse trials (each with

five plants per genotype) and one field trial (five blocks, one row per genotype per block) were conducted from Dec 2007 to July 2008. Real time PCR quantification was not performed in the 2008 field trial. In the greenhouse, plants subject to the same treatment were randomized within a block in order to eliminate the variance due to environmental factors.

7. Data analysis

Mixed model analyses were performed in JMP 7.0 for the field trials following an alpha design. For trials conducted in one field location, “maize lines” was specified as a fixed factor, whereas “replications” and “blocks within replications” were specified as random. Data from the 2006 trials in NY and NC were analyzed separately because of the different field arrangements and disease rating scales. A combined analysis across two locations (NY and NC) was conducted for the 2007 trials by fitting a mixed model with “maize lines”, “locations” and “maize lines by locations” as fixed factors, and “replications within locations” and “blocks within replications within locations” as random effects. Phenotypic differences between TBBC3 lines/NILs and B73, or between any two NILs, were determined by pair-wise comparisons of least squares means using two-tailed Student's t-test at $P < 0.05$. Correction for multiple comparisons was not made since all the tests were independent. The TBBC3 lines and derived NILs were each compared to the recurrent parent B73, and pairs of NILs were only compared to each other when sharing identical genotypes except for the target introgression(s) under testing. Overall comparisons of TBBC3 lines or NILs were not intended in this analysis.

The QTL effect of each marker locus was investigated in the full TBBC3 population. Considering the presence of unlinked potentially unrecognized introgressed segments in the same lines, a series of statistical tests was conducted as

described by Szalma et al. (2007) with some modification. Markers that are likely associated with resistance ($P < 0.05$) were identified by: 1) comparing the least squares mean of TBBC3 lines homozygous for Tx303 alleles at each locus to the least squares mean of B73 rows, and 2) performing one-way ANOVA (analysis of variance) on an individual marker – trait basis in the TBBC3 population. Significant markers were grouped into linked introgression blocks, and the markers with the greatest significance in each linked block were considered to reflect the most likely QTL position. Correlations between the most significant markers were checked. For each pair of unlinked but correlated markers, TBBC3 lines fixed for one marker were grouped and analyzed for the co-segregation of the second marker and disease traits, using two tailed Student's *t* test at $P < 0.05$. A putative QTL was declared if the mean of all the lines with Tx303 alleles at this locus was significantly different from B73, and significant difference was detected for lines contrasting for this locus under the condition of another potential QTL locus being fixed.

In segregating populations, the phenotypic and genotypic data were first analyzed by mixed stepwise regression, with a significance probability of $P < 0.05$ for each parameter to enter/leave the model (van Dam et al. 2003). Once the markers significantly associated with the traits were identified, analysis of variance (ANOVA) was conducted on a single marker basis at $P < 0.05$ to estimate QTL effects. For the populations with more than one marker showing significant effect on traits, the effective markers were further tested in pairs by two-way ANOVA for their epistatic interactions. Allele effects were determined by pairwise two-tailed Student's *t* test according to the least significant difference (LSD) at $P = 0.05$.

The proportion data from microscopic analysis and quantitative PCR were transformed to arcsine of the square roots prior to statistical analysis. Arcsine transformed data were analyzed by fitting a linear least squares model with

“genotype” and “replicate” as two independent variables, and with the number in the denominator of each proportion as a weighting factor. For quantitative PCR, considering the biological and technical replicates, the fungal biomass ratios were also arcsine square-root transformed, but were analyzed with a different linear least squares model of $y_{ijm} = \beta_0 + \beta_1 (\text{genotype}_i) + \beta_2 (\text{rep}_j) + \beta_3 (\text{plate}_m (\text{rep}_j)) + e_{ijm}$ (genotype i = B73, Tx303 and two derived NILs; rep j = 1, 2, 3; plate m = 1, 2, 3; e_{ijm} : random experimental error). Least squares means and 95% confidence intervals were back-transformed to percentages. Confidence intervals were larger than significance levels due to asymmetry resulting from back transformation of arcsine scale. The significant QTL effects were estimated by pairwise comparisons of least squares means of introgression lines/NILs and B73 using two-tailed Student's t-test at $P < 0.05$. All the statistical analyses in the study were conducted using JMP 7.0.

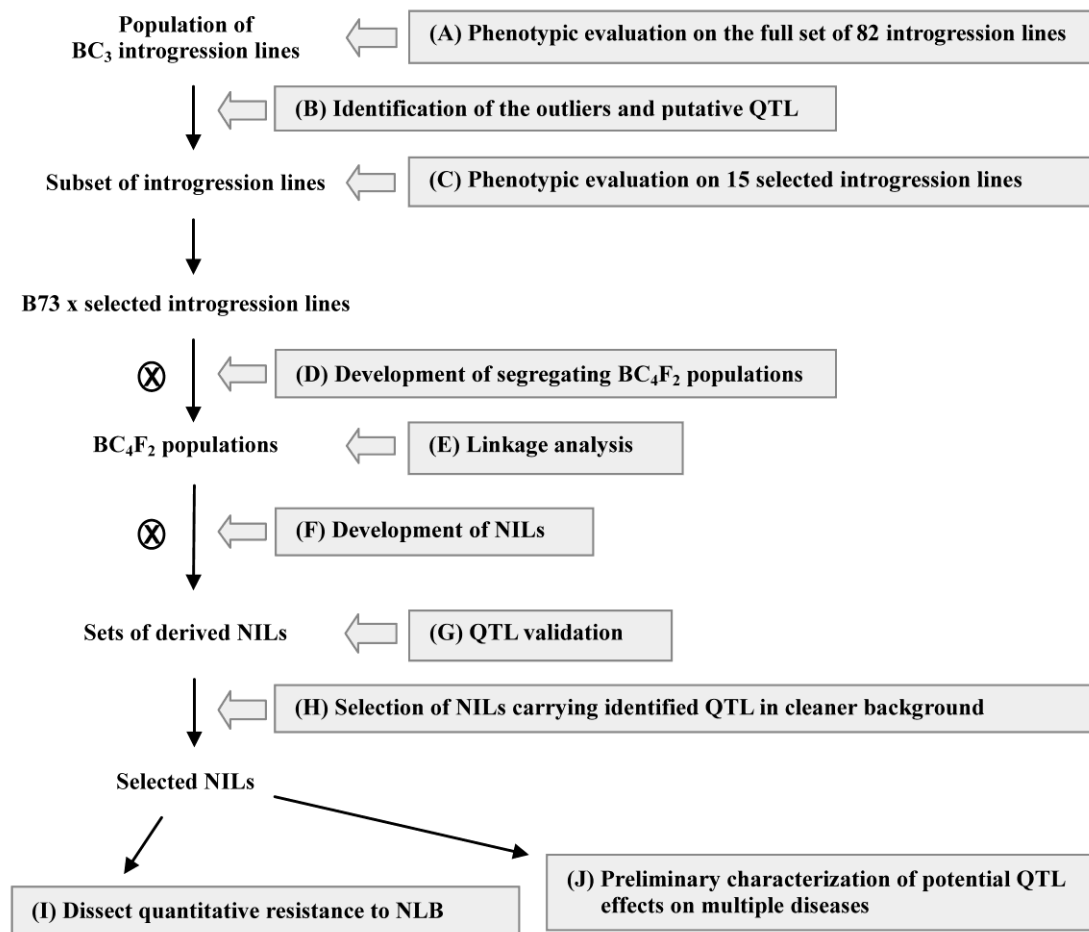
RESULTS

A stepwise strategy (Fig. 1.1) was conducted to map and characterize QTL using the population of TBBC3 introgression lines and its derived NILs.

1. Identification of outliers and putative NLB QTL in the TBBC3 population

The full set of 82 TBBC3 lines was screened for NLB resistance at field sites in New York and North Carolina in 2006. As shown in Fig. 1.2, the TBBC3 population was generally more susceptible than B73 at both locations. Lines carrying loci affecting NLB resistance were identified based on their phenotypic differences from the B73 recurrent parent. In the trial at Aurora NY, three lines were scored as significantly more resistant and 20 lines were scored as significantly more susceptible than B73 for PrimDLA, while no lines showed significantly more resistance and 39 lines showed more susceptibility than B73 based on AUDPC. In the trial at Clayton NC, eight lines were significantly more resistant and 13 lines were significantly more

Figure 1.1 Strategy for mapping and characterizing QTL using introgression lines. (A) Initial screening was conducted at Aurora NY and Clayton NC, with two replications per location. Conventional disease components, including incubation period (IP), diseased leaf area (DLA), and disease severity were evaluated. (B) Lines that differed from B73, and lines showed relatively extreme phenotypes in the population were determined. Putative QTL were also identified. (C) Lines carrying putative NLB QTL were evaluated to confirm their differential resistance/susceptibility relative to B73. Plants were tested at the juvenile (two locations, with four replications each) and adult (one location, with three replications) stage for IP, LE, DLA and AUDPC. This allowed the test of *Hypothesis #2: the effectiveness of disease QTL is affected by plant maturity*. (D) Selected lines were backcrossed to B73, then selfed to generate F₂ populations segregating for the introgressed regions within the populations. (E) Individual plants were tested for markers targeting introgressed regions, and cosegregation was assessed with the disease components measured in the greenhouse or field in NY. Candidate NLB QTL were determined if trait-marker association was detected in more than one F₂ population. (F) The F₃ and/or F₄ near-isogenic lines (NILs) carrying different Tx303 introgressions were derived from F₂ populations. (G) The effects of different introgressions on conventional disease components were further tested (two replications at Aurora NY). The NLB QTL were declared if the QTL effects were validated in the NILs. (H) Selected NILs carrying identified QTL were then used for detailed QTL characterization. (I) Selected NILs were evaluated with a series of disease components targeting different stages of disease development (Table 1) in the greenhouse (three replications) and field (one replication). This allowed the tests of *Hypothesis #1: individual QTL affect distinct stages of disease development* and *Hypothesis #3: the effectiveness of QTL is affected by environmental conditions*. (J) Selected NILs were also evaluated for anthracnose stalk rot, common rust, common smut and Stewart's wilt at Aurora NY. The one-year result provided preliminary insight regarding *Hypothesis #4: QTL for resistance to NLB comprises genes or gene clusters involving in broad-spectrum resistance*.



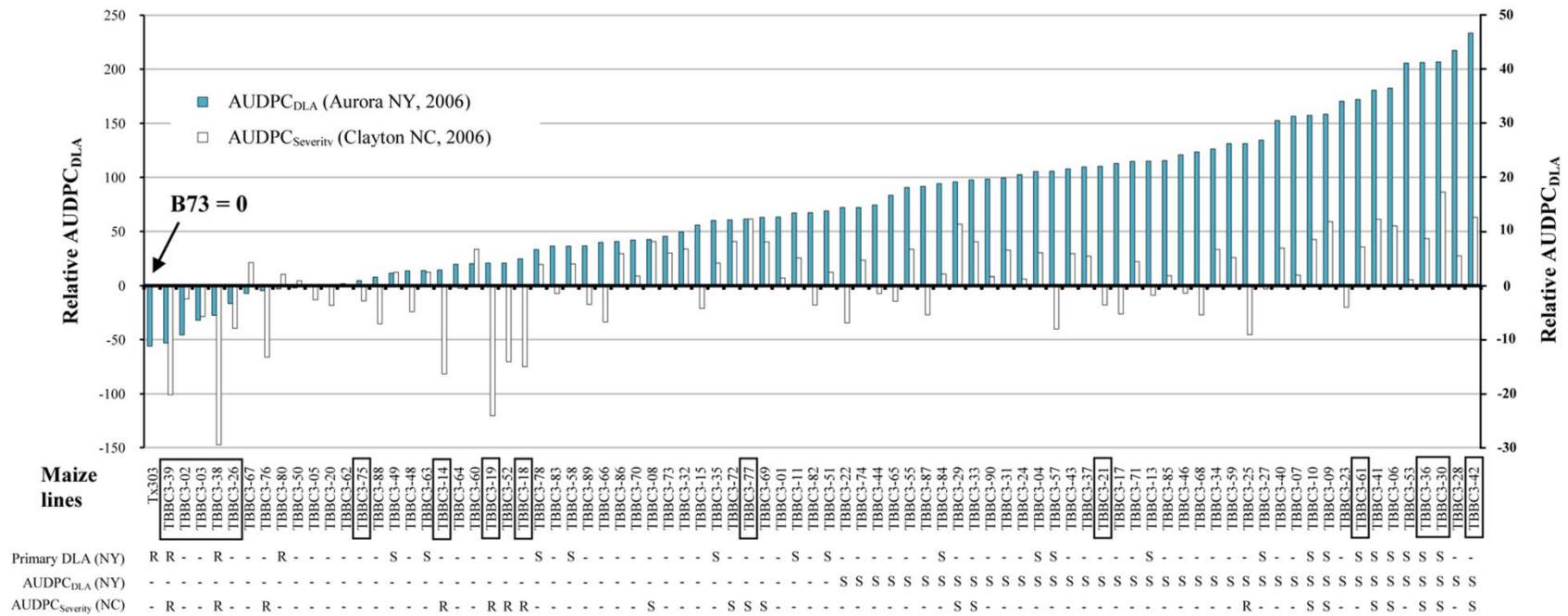


Figure 1.2 NLB resistance of the full set of 82 TBBC3 introgression lines. Relative AUDPC values shown are the differences of least squares means (from mixed models) between TBBC3 lines and B73 recurrent parent. AUDPC_{DLA} (solid bars) was from three DLA scores in the 2006 trial in NY, and AUDPC_{Severity} (open bars) was derived from three severity scores in the 2006 trial in NC. In NY, primary DLA was also rated for diseased leaf area on inoculated leaves. The letters “R” and “S” below the graph indicate the lines significantly more resistant and more susceptible than B73, respectively, based on primary DLA, AUDPC_{DLA} and AUDPC_{Severity}. The 15 TBBC3 lines selected for subsequent phenotypic validation are indicated by rectangles highlighting the maize line designation.

susceptible than B73 for AUDPC. The only line that showed contrasting phenotypes in the two environments was TBBC3-25, which carried Tx303 introgressions at bins 4.06-4.07, 7.01-7.03, 7.04 and 7.05. This line was scored as significantly more susceptible than B73 in New York, but significantly more resistant than B73 in North Carolina, suggesting experimental error or environmental influence on the expression of NLB QTL.

A combination of analytic approaches was used to associate the resistance or susceptibility effects with specific chromosomal segments (Szalma et al. 2007). Based on this analysis, 13 putative NLB QTL were identified in the TBBC3 population (Table 1.2). The result suggested that Tx303 alleles at bins 1.06, 1.11, 3.06 5.08-5.09, and B73 alleles at bins 1.01-1.02, 4.03, 4.07, 5.02, 5.04, 7.03 and 8.03 confer resistance to NLB. Except for bins 1.01 and 5.08-5.09, the putative QTL are co-localized with NLB QTL previously detected in the four maize mapping populations previously reported in the literature.

2. Phenotypic evaluation on the subset of 15 TBBC3 lines

A subset of 15 TBBC3 lines was selected for further evaluation. This selection was based on the degree of difference in NLB resistance between each individual TBBC3 line and B73 at the two field sites. It was also influenced by the identity of the Tx303 introgressions carried by the lines; those carrying introgressions corresponding both to putative QTL identified in TBBC3 population (Table 1.2), and to previously reported NLB QTL (Wisser et al. 2006) were given some priority because we were more interested in QTL validation and characterization than in QTL discovery. In 2007, the 15 selected lines were evaluated at the same field locations in NY and NC to confirm their disease phenotypes. Significant “line” effects ($P < 0.0001$) were found for IP, all the DLA ratings, and AUDPC in each of NY and NC, and across two

Table 1.2 Putative QTL for northern leaf blight (NLB QTL) affecting incubation period (IP), primary diseased leaf area (PrimDLA), diseased leaf area (DLA), disease severity (severity), AUDPC_{DLA} and AUDPC_{Severity} (area under the disease progress curve calculated from DLA or severity) in the TBBC3 introgression lines. QTL effects for each marker locus are the significant differences of least squares means of Tx303 homozygous genotypes at the locus relative to B73 recurrent parent line (* 0.01 < P < 0.05, ** 0.001 < P < 0.01, *** P < 0.001). Putative QTL are reported as correlated groups because of the high dependencies among those introgressed segments in TBBC3 lines. Further tests are required to determine the causal QTL.

Chr. Bin	Marker	Map Position ^a	Resistance allele	Aurora NY, 2006						Clayton NC, 2006				Previously reported NLB QTL at the locus	
				IP (days)	Prim DLA (%)	DLA1 (%)	DLA2 (%)	DLA3 (%)	AUDPC _{DLA} (%-day)	Severity1 (scale)	Severity2 (scale)	Severity3 (scale)	AUDPC _{Severity} (scale-day)	Mapping population ^b	Reference
Correlated loci															
1.01	<i>umc1071</i>	85.2	B73		6.6*		5.5***	8.0***	110.1***	0.7**				None	
1.02	<i>bnlg1429</i>	143.5	B73		6.9*	6.2***	7.1***	7.4**	134.7***					<u>B52</u> x Mo17	Freymark et al. 1993
	<i>bnlg1953</i>	170.0	B73		7.9**		6.3***	9.3***	120.0***	0.7**					
4.07	<i>bnlg1621</i>	349.6	B73		5.0*	3.0**	4.9***	9.4***	91.8***					D32 x <u>D145</u>	Welz et al. 1999b
5.02	<i>bnlg565</i>	150.9	B73		4.6*	2.7***	4.9***	10.0***	90.7***					B52 x <u>Mo17</u>	Dingerdissen et al. 1996
															Freymark et al. 1993
7.03	<i>bnlg434</i>	323.3	B73		6.6*	3.8***	4.7***	4.1*	84.0***					B52 x <u>Mo17</u>	Dingerdissen et al. 1996
															Freymark et al. 1993
8.03 ^d	<i>UMC32B</i>	199.1	B73			8.8***	12.9***	23.5***	246.0***	1.3**			13.2**	None	
Correlated loci															
1.06 ^c	<i>umc2234</i>	529.0	Tx303							-1.1*			-12.2*	<u>D32</u> x D145	Welz et al. 1999b
5.08 ^d	<i>umc1225</i>	641.4	Tx303	1.6**	-18.6**					-2.0**	-1.9***	-1.2**	-31.9***	None	
	<i>bnlg1695</i>	664.2	Tx303	1.6**	-18.6**					-2.0**	-1.9***	-1.2**	-31.9***	None	
5.09 ^d	<i>umc1829</i>	671.5	Tx303	1.6**	-18.6**					-2.0**	-1.9***	-1.2**	-31.9***	None	

Table 1.2 (Continued)

Chr. Bin	Marker	Map Position ^a	Resistance allele	Aurora NY, 2006						Clayton NC, 2006				Previously reported NLB QTL at the locus	
				IP (days)	Prim DLA (%)	DLA1 (%)	DLA2 (%)	DLA3 (%)	AUDPC _{DLA} (%-day)	Severity1 (scale)	Severity2 (scale)	Severity3 (scale)	AUDPC _{Severity} (scale-day)	Mapping population ^b	Reference
Correlated loci															
4.03	<i>umc2082</i>	141.6	B73		11.6***		7.4***	12.4***	132.9***	0.6*	0.4*		7.4*	D32 x <u>D145</u>	Welz et al. 1999b
	<i>umc2176</i>	174.6	B73		11.6***		7.4***	12.4***	132.9***	0.6*	0.4*		7.4*		
5.04	<i>bnlg1208</i>	323.1	B73	-0.6*	15.4***	3.3**	6.3***	7.4**	103.8***	0.8*	0.5*	0.4*	10.2**	Lo951 x <u>CML202</u>	Schechert et al. 1999 Welz et al. 1999a
														D32 x <u>D145</u>	Welz et al. 1999b
Correlated loci															
1.11 ^d	<i>bnlg131</i>	1065.6	Tx303							-1.5***	-0.9*		-16.5**	IL731a x <u>W6786</u>	Brown et al. 2001 Schechert et al. 1999
														Lo951 x <u>CML202</u>	Welz et al. 1999a
3.06 ^d	<i>bnlg2241</i>	452.7	Tx303							-1.5***	-0.9*		-16.5**	<u>IL731a</u> x <u>W6786</u>	Brown et al. 2001 Freymark et al. 1993
														B52 x <u>Mo17</u>	Schechert et al. 1999
														<u>Lo951</u> x <u>CML202</u>	Welz et al. 1999a

^a The marker position was based on genetic map of intermated B73 x Mo17 population (version IBM 2008 neighbors).

^b The mapping population in which the same locus was detected for resistance to NLB. The resistance donor was underlined.

^c This marker was first found highly associated with NLB resistance in segregating populations B73 x TBBC3-38 and B73 x TBBC3-39. It was then tested in 82 TBBC3 lines.

^d Introgressed segment at the locus was only present in a single TBBC line (bin 8.03 in TBBC3-42; bin 5.08-5.09 in TBBC3-38; bin 1.11 and 3.06 in TBBC3-30).

locations. The genotype effect was also significant ($P < 0.0001$) for LE (tested in only NY). In the combined analysis across environments, significant effects of “location” ($P < 0.002$ for IP, DLA and AUDPC) and “line-by-location” ($P < 0.0001$ for DLA and AUDPC) were also detected. The variations were attributed to dissimilar environmental conditions and disease ratings conducted by different scorers at two sites. Inspection of individual TBBC3 lines in NY and NC respectively suggested that most of the lines performed similarly across the two locations. Pairwise comparisons validated the phenotypic differences in seven out of the 15 TBBC3 lines and B73. Consistently in NY and NC, TBBC3-38 and TBBC3-39 were more resistant, whereas TBBC3-42, TBBC3-36, TBBC3-21, TBBC3-30, and TBBC3-77 were more susceptible than B73.

Three selected TBBC3 lines showed different degrees of resistance or susceptibility in two field environments in both 2006 and 2007 trials (Fig. 1.2 and Table 1.3). TBBC3-19 was significantly more resistant than B73 for IP ($P = 0.03$), DLA1 ($P = 0.001$), DLA2 ($P = 0.035$) and AUDPC ($P = 0.010$) in NC but not NY. In contrast, TBBC3-61 was found significantly more susceptible than B73 for three DLA ratings and AUDPC ($P < 0.0001$) in NY but not in NC. The greater susceptibility of TBBC3-14 relative to B73 was only observed in NY for three DLA ratings ($P < 0.003$) and AUDPC ($P < 0.0001$; significance levels refer to the 2007 trials).

Ten selected TBBC3 lines showed significantly greater days to anthesis (DTA) than B73 (about 1-4 days of difference, Table 1.3), indicating the presence of flowering time QTL in those lines. A significant correlation between flowering time and NLB resistance has been observed in a set of diverse maize lines (R. Wisser, J. Kolkman, and P. Balint-Kurti, pers. comm.), suggesting some epistatic and/or pleiotropic interaction between NLB QTL and flowering time QTL.

To test the effect of plant maturity on QTL expression, the 15 selected lines were

Table 1.3 NLB resistance of the subset of 15 TBBC3 introgression lines. In 2007, the same set of plant materials was inoculated at six-leaf stage in New York (NY) and North Carolina (NC), and was grown in a separate field plot in NY and inoculated 1-2 weeks before anthesis. Trait values shown are the least squares means of maize lines relative to the recurrent parent B73. Phenotypes that were significantly more resistant than B73 are highlighted in bold and shaded, while the phenotypes significantly more susceptible than B73 are underscored. The significance level was determined by pairwise two-tailed Student's t test on the difference between least squares means (denoted as * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$). Genotypes in bold are the ones that were consistently more resistant or more susceptible than B73 across two field environments.

Maize line	Introgressed region (Bin) ^a	Days to anthesis	Trait (juvenile plants being inoculated; NY and NC) ^b			Trait (adult plants being inoculated; NY)	
			Relative IP	Relative LE	Relative AUDPC	Relative IP	Relative AvgDLA ^d
Tx303		8.7***	2.1***	-0.3	-164.1***	7.5***	-8.7***
TBBC3-38	1.03, 1.06, 5.00, 5.03, 5.07-5.09	4.0***	2.1***	-0.1	-110.2***	2.6***	-4.5***
TBBC3-39	1.03, 1.06, 5.00, 5.07	2.4***	0.9*	0.0	-67.2**	1.3*	-4.3***
TBBC3-18	1.11, 7.05, 7.06, 10.03, 10.04	4.2***	0.8*	-0.1	-35.8	1.3*	-2.0**
TBBC3-19 ^c	7.04, 7.06, 9.03, 10.04	1.4*	1.2**	0.1	-29.0	1.2	<u>3.2***</u>
TBBC3-26	7.06	1.0	1.4**	0.0	-1.8	-0.1	1.0
TBBC3-75 ^d	6.04-6.05	1.2	0.4	0.1	12.2	2.6***	-2.3**
TBBC3-02	1.07-1.08, 5.01-5.02, 8.03-8.05	3.2***	-0.7	0.2	35.7	0.6	<u>1.5*</u>
TBBC3-14 ^c	1.09-1.10, 4.07	3.1***	<u>-0.9*</u>	0.0	<u>67.0**</u>	0.3	<u>6.7***</u>
TBBC3-03	1.01, 1.04, 7.03, 8.03-8.05	2.8***	-0.1	-0.1	<u>117.0***</u>	<u>-1.4*</u>	<u>5.7***</u>
TBBC3-61 ^c	1.10	1.4*	-0.5	<u>0.6***</u>	<u>126.7***</u>	0.2	<u>7.0***</u>
TBBC3-77	1.01-1.03, 6.04-6.05	4.3***	<u>-1.4**</u>	<u>0.9***</u>	<u>139.4***</u>	<u>-1.4*</u>	<u>4.0***</u>
TBBC3-30	4.02-4.03, 5.04	0.2	<u>-0.9*</u>	<u>0.6***</u>	<u>195.4***</u>	-0.6	<u>4.7***</u>
TBBC3-21	4.07, 9.01-9.04	0.6	<u>-1.7***</u>	<u>0.8***</u>	<u>275.5***</u>	-1.1	<u>7.8***</u>
TBBC3-36	1.01, 1.03-1.05, 4.01, 8.08	1.3	-0.8	<u>0.7***</u>	<u>305.6***</u>	<u>-1.8**</u>	<u>8.2***</u>
TBBC3-42	1.01-1.02, 4.07-4.08, 5.01-5.02, 8.02-8.05	2.2**	<u>-2.1***</u>	<u>0.7**</u>	<u>310.8***</u>	<u>-1.3*</u>	<u>8.7***</u>

^a Introgressed genome regions from Tx303 (heterozygous regions not shown). Bin position was based on genetic map of intermated B73 x Mo17 population (version IBM 2008 neighbors).

^b Relative least squares means of IP and AUDPC shown were from the combined analysis across the trials in NY and NC. LE was only scored in NY.

^c Individual TBBC3 lines in each field environment were inspected. TBBC3-19 was significantly more resistant than B73 only in NC. TBBC3-14 and TBBC3-61 showed greater susceptibility than B73 only in NY.

^d Resistance to NLB was more effective in adult plants.

inoculated at juvenile and adult stages (Table 1.3) and their disease traits were compared. It was assumed that variable maturity would have no effect on juvenile phenotypes. In all the lines, QTL effects in reducing or increasing resistance to NLB were generally consistent at juvenile and adult plant stage. No lines were observed to show contrasting phenotypes (more resistant and more susceptible relative to B73) when inoculated at six-leaf stage or just before flowering, which suggested that allele effects of NLB QTL were not altered by plant maturity. There was, however, evidence suggesting that the effectiveness of QTL might change over plant development. The resistance in TBBC3-38, TBBC3-39, TBBC3-18 and TBBC3-75 was more effective in adult than in juvenile individuals. The formation of NLB lesions was delayed by around 0.5-2 days on mature plants of the same genotypes.

3. Validation of QTL effects by linkage analysis

Each of the TBBC3 lines had more than one identified introgression from Tx303. To determine which of the introgressed regions in a given line was associated with resistance, several segregating BC₄F₂ populations were developed from crosses between selected TBBC3 lines and the recurrent parent B73. The BC₄F₂ individuals from each cross were evaluated for NLB resistance and genotyped using markers for the respective introgressed regions. TBBC3 lines used for generating BC₄F₂ populations included TBBC3-02, TBBC3-38, TBBC3-39, TBBC3-42, and TBBC3-77.

Significant trait-marker association was identified in all the BC₄F₂ populations under investigation (Table 1.4). The segregating populations were analyzed for trait-marker association in the greenhouse during winter 2006-2007, and/or at Aurora NY in 2007. For further confirmation and characterization of QTL, two sets of near-isogenic lines (BC₄F₃ and BC₄F₄) containing different Tx303 introgressions from the original selected lines were derived from lines of interest. The BC₄F₃ and BC₄F₄ NILs

Table 1.4 Validation of NLB QTL in the BC₄F₂ segregating populations. The trait-marker association was tested by ANOVA at $P < 0.05$. Introgressions/markers significantly associated with NLB resistance are listed. The relative allele effects are the differences on the least squares means between Tx303 homozygous genotypes and B73 homozygous genotypes at the locus.

Introgressed region (Chr. bin) ^a	Map interval (cM) ^b	SSR marker (map position, cM) ^b	Parental donor lines of the BC ₄ F ₂ population ^c	Environment (location, year)	Resistance allele	R^2	Relative allele effect (LSMean _{Tx303} – LSMean _{B73}) ^d					
							IP (dpi)	LE (mm/day)	DLA1 (%)	DLA2 (%)	DLA3 (%)	AUDPC (%-day)
1.06 [§]	487.5-590.5	umc2234 (529.0)	TBBC3-38	Aurora NY, 2006	Tx303	0.330 ^f	1.96**	-0.29*	-6.36***	-6.69***	-5.31***	-119.27***
1.06 [§]	487.5-590.5	umc2234 (529.0)	TBBC3-39	Aurora NY, 2006	Tx303	0.526 ^f	2.38***	-0.33**	-5.79***	-8.39***	-9.54***	-151.70***
1.01-1.02 [§]	0-184.2	bnlg1953 (170.0)	TBBC3-42	Greenhouse, 2006	B73	0.139 ^e	-0.60***	0.35*	na	na	na	na
1.01-1.02 [§]	0-184.2	bnlg1953 (170.0)	TBBC3-77	Aurora NY, 2006	B73	0.074 ^f	-0.58	0.11	6.05***	3.36*	1.42	70.95*
5.00	0-30.0	mmc0151 (20.5)	TBBC3-77	Aurora NY, 2006	B73	0.059 ^f	-0.71	0.26	4.30**	4.37**	3.04*	80.37**
5.01-5.02	115.1-153.9	bnlg565 (150.9)	TBBC3-02	Aurora NY, 2006	B73	0.053 ^e	-1.30**	0.03	1.98	0.87	0.52	21.13
5.08-5.09	603.0-676.1	bnlg1829 (313.3)	TBBC3-38	Aurora NY, 2006	Tx303	0.046 ^f	0.71	0.01	-2.02	-2.45*	-3.67*	-49.85**
8.03-8.05	265.2-344.0	umc1130 (330.1)	TBBC3-02	Aurora NY, 2006	Tx303	0.114 ^f	0.41	-0.04	-2.97**	-3.90***	-3.02***	-68.90***

[§] The QTL effects on NLB resistance were further confirmed in derived BC₄F₃ and/or BC₄F₄ NILs.

^a Introgressed regions that were significantly associated with traits in the segregating populations. The genome regions are shown as bin positions (eg. bin 1.06 is the sixth segment in maize chromosome 1).

^b SSR marker with the most significant effects on traits. The marker position was based on genetic map of intermated B73 x Mo17 population (version IBM 2008 neighbors). The map interval of each introgression is assumed to extend halfway between two markers around the border of each end of the introgression.

^c The selected TBBC3 lines used for developing the segregating BC₄F₂ populations. The lines were backcrossed to B73 and selfed. Phenotyping and genotyping were conducted on individual plants in the populations.

^d The significance level was determined by pairwise two-tailed Student's t test on the difference between least squares means (denoted as * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$).

^e R -square from the ANOVA using IP as the dependent variable, referring the proportion of the variation in IP accounted for by the marker in the third column.

^f R -square from the ANOVA using AUDPC as the dependent variable, referring the proportion of the variation in AUDPC accounted for by the marker in the third column.

were evaluated at Aurora, NY for NLB in 2007 and 2008. Responses to other diseases, including rust, anthracnose stalk rot, Stewart's wilt, and smut, were assessed in 2008.

The QTL at bin 1.02 was found effective in two segregating populations: those derived from TBBC3-42 and TBBC3-77. Although the introgressed region extended from bin 1.01-1.02, higher significance was detected at the marker located at bin 1.02 so the QTL was designated *qNLB1.02*. In the BC₄F₂ derived from TBBC3-42, ~14% of the IP variation could be attributed to *qNLB1.02*. The B73 allele at *qNLB1.02* was associated with slower disease development. Individuals homozygous for Tx303 alleles in bin 1.02 (loss of *qNLB1.02*_{B73}) were significantly more susceptible than B73. The Tx303 homozygotes showed ~0.6 days of lower IP ($P < 0.0001$) and ~0.35 mm/day of greater LE ($P < 0.05$) in the B73 x TBBC3-42 population. The same QTL acted differently in the B73 x TBBC3-77 population. While *qNLB1.02* was effective in reducing DLA and AUDPC, it did not affect IP in the B73 x TBBC3-42 population. It is worth noting that TBBC3-42 is a much more susceptible genotype than TBBC3-77, which provided a better backdrop for evaluating minor disease QTL.

Two derived NILs carrying identified NLB QTL in "cleaner" B73 backgrounds (minimal amount of introgression from Tx303) were chosen for detailed QTL characterization. TBBC3-38-05F was a BC₄F₃ NIL with a Tx303 introgression at bin 1.06 (designated as the NIL with *qNLB1.06*_{Tx303}), and TBBC3-42-10E-02 was a BC₄F₄ NIL with a Tx303 introgression at bin 1.02 (designated as the NIL with *qNLB1.02*_{Tx303}). To investigate the effect of QTL during NLB development, a series of disease components (Table 1.1) was selected or developed and applied on the two derived NILs in three replicated experiments in the greenhouse during Dec 2007 – Apr 2008, and at Aurora, NY during summer 2008.

The QTL at bin 1.06 was not identified based on previously reported genotype information. Although clear phenotypic variation was observed on IP and DLA in

the BC₄F₂ populations from TBBC3-38 and TBBC3-39, no significant association was found between IP and the known introgressions at bins 1.03, 5.00, 5.01-5.03, and 5.07-5.09. The IP on BC₄F₂ individuals of TBBC3-38 and TBBC3-39 ranged from 11 dpi to 18 dpi, while IP on B73 was generally between 12 and 15 dpi. Since these two populations displayed a greater variation in resistance, we inferred that a disease QTL was present in the population but not associated with a recognized introgression. To search for unrecognized introgressions, an additional 68 SSR markers across the genome were chosen to target chromosomal regions that were not well-covered by the original 130 RFLP and SSR markers on TBBC3 map. Pooled DNA samples from BC₄F₂ individuals of TBBC3-38 and TBBC3-39, along with B73 DNA as a control, were tested with the additional SSR markers for heterozygosity. Among the 68 SSRs tested, *umc1754* and *umc2234* (both in bin 1.06) were the only polymorphic markers segregating in both BC₄F₂ populations. This indicated the presence of a Tx303 introgression at bin 1.06 in both TBBC3-38 and TBBC3-39. Individuals in each population were then genotyped with *umc1754* and *umc2234*, and analyzed for the effects on various disease components. A highly significant association was detected for various parameters in both populations (Table 1.4). In the B73 x TBBC3-38 and B73 x TBBC3-39 populations, around 33% and 53% (respectively) of the variation in AUDPC was explained by this QTL. Compared to the B73 allele, the Tx303 allele at *qNLB1.06* increased the incubation period by ~2 days, decreased lesion expansion by ~0.3 mm per day, and reduced DLA by about 5-10 %. Another QTL at bins 5.08-5.09 (Tx303 allele for resistance) was found in the B73 x TBBC3-38 population. The effect was relatively minor ($R^2 \approx 0.05$) compared to the *qNLB1.06*_{Tx303} identified in the same population.

QTL were also identified in bins 5.00, 5.01-5.02, and 8.03-8.05. Although contributing significant level of resistance for IP or DLA in the B73 x TBBC3-77 and

B73 x TBBC3-02 populations, the same introgressions/markers were not found effective in the populations derived from TBBC3-38, TBBC3-39 and TBBC3-42. The ambiguous results called the significant association into question, suggesting that the effectiveness of QTL at bins 5.00, 5.01-5.02, and 8.03-8.05 was affected by genetic background and/or environmental conditions.

4. Validation of *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}* in selected NIL sets

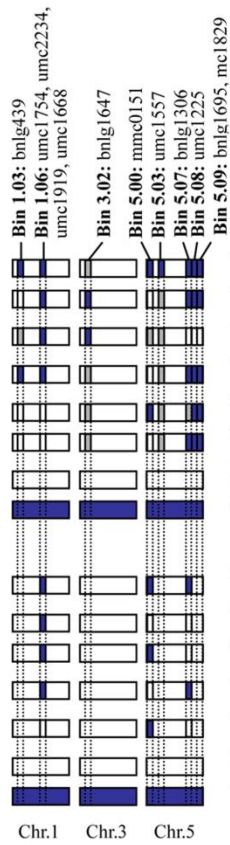
To confirm the QTL effects detected in segregating populations, NILs were generated by selfing selected BC₄F₂ lines from the populations B73 x TBBC3-38, B73 x TBBC3-39 and B73 x TBBC3-42. Selected lines were chosen to represent different introgressed regions in the original TBBC3 lines. The QTL effects were determined by pairwise comparisons between individual introgression lines and B73, and between the NILs developed from a TBBC3 line. Within each set of NILs, lines contrasting for each marker locus were grouped and analyzed for their phenotypic differences. The NILs derived from different TBBC3 lines were not compared to each other since the phenotypic difference can be attributed to not only introgressed regions but also to minor differences in genetic backgrounds.

Evaluations of the BC₄F₃ NILs validated the effects of *qNLB1.06_{Tx303}*. Five NILs derived from B73 x TBBC3-38 and four NILs from B73 x TBBC3-39 were phenotyped in 2008. Individual trait-locus analysis in the two NIL sets revealed that bin 1.06 was the only locus that showed significant effects on resistance. As shown in Table 1.5, most of the NILs carrying Tx303 alleles at bin 1.06 were significantly more resistant than B73. The only exception was the line TBBC3-39-11A, which might have lost the resistance gene(s) due to recombination. Significant differences between lines with *qNLB1.06_{Tx303}* and B73 were observed for IP, DLA and AUDPC, which was in agreement with the results from linkage analysis in BC₄F₂ populations (Table. 1.4).

The effects of *qNLB1.06_{Tx303}* relative to *qNLB1.06_{B73}* on IP (1.5-2.5 days) and DLA (2-10% through the season) were determined by comparing TBBC3-38-05F with B73 and TBBC3-39-19E with B73. The significant effect of reducing lesion expansion was not confirmed in the NILs. Lines with *qNLB1.06_{Tx303}* even showed significantly higher LE scores, indicating some variations from different genetic backgrounds and environments.

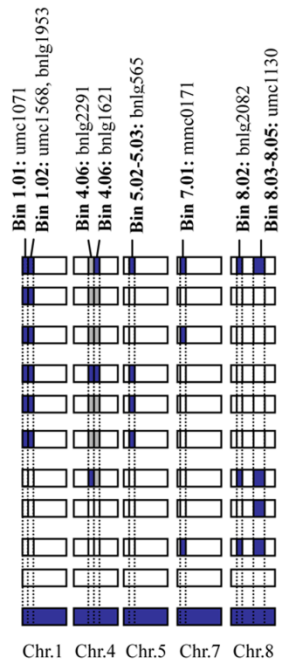
The QTL effect at bin 5.08-5.09 was detected in the B73 x TBBC3-38 population, but was not confirmed in the NILs. Two lines with Tx303 introgression at bin 5.08-5.09, TBBC3-38-16G and TBBC3-38-07H, did not show higher levels of resistance than B73 for all the disease components. In fact, TBBC3-38-16G was significantly more susceptible than B73, suggesting either that the B73 allele at 5.08-5.09 contributed resistance (as opposed to the indication of Tx303 allele for resistance in previous linkage analysis), or the segregation of background introgression(s). Other introgressions segregating in the NILs included bins 1.03, 5.00 and 5.07. Consistent with the linkage analysis in BC₄F₂ populations, bin 5.07 was not effective for NLB resistance in the NILs (same level of disease in TBBC3-39 and TBBC3-39-08F). The QTL effect of bin 5.00 was unclear because of contradictory results resulting from pairwise comparisons. TBBC3-38 was significantly more resistant than TBBC3-38-18A, suggesting that Tx303 allele(s) at bin 5.00 contributed to resistance. However, TBBC3-39-09F was significantly more susceptible than B73, indicating that the resistance effect was contributed by B73 allele(s). Moreover, no significant differences were consistently seen for any disease parameters for TBBC3-39-08F and TBBC3-39-19E, which differed at bin 5.00. The effect of bin 1.03 was also ambiguous. The B73 allele at bin 1.03 showed some minor but significant effects on DLA3, DLA4 and AUDPC, based on the comparison between TBBC3-38-11E and TBBC3-38-18A, but the effectiveness was not detected in the BC₄F₂ population.

Table 1.5 Genotypes and disease phenotypes for Tx303, B73 and the NIL sets derived from B73 x TBBC3-38 and B73 x TBBC3-39. Among all the target introgressions including bins 1.03, 1.06, 3.02, 5.00, 5.02-5.03 and 5.07-5.09, only *qNLB1.06_{Tx303}* (Tx303 allele at bin 1.06) was validated for resistance to NLB. The open bars and solid bars represent the loci homozygous for B73 alleles and Tx303 alleles, respectively. The gray bars represent heterozygous loci or missing genotypic data. Only the chromosomes with introgressed regions in the two NIL sets are shown. The rest of the genome was assumed fixed for B73 alleles. Trait values are least squares means calculated from the mixed model. Pairwise Student's t tests were performed to analyze the differences between each NIL and B73, and between every pair of NILs in each set. Trait values with different letters are significantly different from each other. Disease phenotypes that were significantly more resistant than B73 are highlighted in bold and shaded, while the phenotypes significant more susceptible than B73 are underscored. Lines that showed significantly different days to anthesis are in bold italic. *qNLB1.06_{Tx303}* was also effective for resistance to Stewart's wilt.



Northern leaf blight							Stewart's wilt	Anthracnose stalk rot	Common smut		Common rust	Anthesis
IP	LE	DLA1	DLA2	DLA3	DLA4	AUDPC	PrimDLA	IN1-6	Ear gall	Stalk gall	AUDPC	Days to anthesis
17 A	1.8 DEFG	10 H	13 I	18 IJ	20 GH	462 J	0 N	174 A	0 BC	0 B	53 H	78 B
15 ABC	1.5 GH	15 DEFG	14 HI	19 I	20 GH	501 IJ	16 JKL				64 DEFGH	77 BCDEFGH
15 BCD	1.4 H	10 H	13 I	19 IJ	25 GH	504 IJ	16 KL				70 DEFGH	75 EFGHI
14 CDEF	1.6 FGH	11 GH	18 FGH	25 EFGH	28 F	637 H	20 IJK				55 GH	78 BCD
15 BCD	1.6 FGH	13 DEFGH	22 DEFG	28 DEFG	38 CD	748 EFG	30 HIJ				59 FGH	76 CDEFGH
13 DEFG	2.0 BCD	21 BC	28 C	29 DEF	34 DE	840 DE	48 DEF	141 CD	1.1 BC	0 B	74 DEF	77 BC
13 F	1.5 H	15 DEF	21 EF	26 GH	33 E	710 G	44 F	142 D	0 C	0 B	61 GH	74 I
14 BCDE	2.0 BCDE	12 EFGH	14 HI	15 J	16 H	427 J	13 KLM	97 E	2.5 A	1.6 A	83 CD	87 A
15 BCD	1.9 CDEF	12 FGH	18 GHI	24 H	29 F	616 H	3 MN	148 CD	0.1 BC	0 B	57 GH	78 B
16 AB	1.6 FGH	13 EFGH	18 FG	19 IJ	23 G	558 HI	5 LMN	141 CD	0.1 BC	0 B	67 DEFGH	75 FGH
14 BCD	1.6 FGH	13 DEFGH	18 GHI	25 FGH	29 F	634 H	16 JKL				61 FGH	76 CDEFGH
12 FGH IJ	1.7 DEFGH	17 CD	26 CD	29 DE	35 CDE	813 DEF	11 KLMN				61 FGH	76 CDEFGH
12 GHIJ	1.8 DEFG	16 DE	27 C	35 C	38 CD	885 D	35 GH	155 ABCD			64 EFGH	75 H
13 F	1.5 H	15 DEF	21 EF	26 GH	33 E	710 G	44 F	142 D	0 C	0 B	61 GH	74 I
14 BCDE	2.0 BCDE	12 EFGH	14 HI	15 J	16 H	427 J	13 KLM	97 E	2.5 A	1.6 A	83 CD	87 A

Table 1.6 Genotypes and disease phenotypes for Tx303, B73 and the NIL set derived from B73 x TBBC3-42. Among all the target introgressions including bins 1.01-1.02, 4.06-4.07, 5.02-5.03, 7.01, 8.02 and 8.03-8.05, only *qNLB1.02_{B73}* (B73 allele at bin 1.02) was validated for resistance to NLB. The open bars and solid bars represent the loci homozygous for B73 alleles and Tx303 alleles, respectively. The gray bars represent heterozygous loci or missing genotypic data. Only the chromosomes with introgressed regions in the NIL sets are shown. The rest of the genome was assumed fixed for B73 alleles. Trait values are least squares means calculated from the mixed model. Pairwise Student's t tests were performed to analyze the differences between each NIL and B73, and between every pair of the NILs. Trait values with different letters are significantly different from each other. Disease phenotypes that were significantly more resistant than B73 are highlighted in bold and shaded, while the phenotypes significant more susceptible than B73 are underscored. Lines that showed significantly different days to anthesis are in bold italic. Preliminary evidence also suggested that *qNLB1.02_{B73}* was effective for resistance to Stewart's wilt, and QTL at bin 1.01-1.02 and/or 5.02-5.03 were associated with resistance to common rust.



TBBC3-42
TBBC3-42-10E-02
TBBC3-42-02E-01
TBBC3-42-06F-02
TBBC3-42-12F-01
TBBC3-42-10E-04
TBBC3-42-05H-04
TBBC3-42-05H-01
TBBC3-42-09E-01
B73
Tx303

Northern leaf blight							Stewart's wilt	Anthracnose stalk rot	Common smut		Common rust	Anthesis
IP	LE	DLA1	DLA2	DLA3	DLA4	AUDPC	PrimDLA	IN1-6	Ear gall	Stalk gall	AUDPC	Days to anthesis
11 HIJ	2.5 A	30 A	45 A	57 A	67 A	1503 A	73 A	154 ABCD	0.5 BC	0 B	106 A	76 CDEFG
11 HIJ	2.3 AB	33 A	46 A	55 A	71 A	1525 A	63 BC	139 CD			104 AB	77 BCDE
11 IJ	2.2 ABC	31 A	45 A	56 A	71 A	1524 A	71 AB	167 ABC			115 A	76 CDEFG
11 J	2.5 A	25 B	39 B	55 A	69 A	1406 B	58 CD	172 AB			84 BCDE	76 CDEFGH
12 HIJ	2.1 ABC	25 B	38 B	49 B	60 B	1287 C	52 DE	148 ABCD			100 ABC	76 CDEFGH
									1.2 B	0.1 B	111 A	77 BCDEF
12 GHJ	1.5 GH	15 DEFG	24 CDE	30 D	40 C	814 DEF	48 DEF	145 BCD			65 DEFGH	75 FGH
13 FGHI	1.5 FGH	15 DEFG	21 EFG	31 CD	37 CD	781 EF	30 HI	146 CD			74 DEFG	76 DEFGH
13 EFGH	1.6 EFGH	15 DEFG	22 DEFG	27 DEFGH	36 CDE	743 FG	45 EFG	142 CD			65 EFGH	75 GH
13 F	1.5 H	15 DEF	21 EF	26 GH	33 E	710 G	44 F	142 D	0 C	0 B	61 GH	74 I
14 BCDE	2.0 BCDE	12 EFGH	14 HI	15 J	16 H	427 J	13 KLM	97 E	2.5 A	1.6 A	83 CD	87 A

qNLB1.02 was validated by analyzing six BC₄F₃ NILs and seven BC₄F₄ NILs derived from a B73 x TBBC3-42 cross. Evaluations conducted in 2007 and 2008 led to similar results. Since BC₄F₄ NILs had cleaner genetic backgrounds, the data from the 2008 trial was shown to represent the overall result. Individual trait-locus analysis in this NIL set suggested that bin 1.01-1.02 was the only locus affecting resistance. The NILs carrying Tx303 allele(s) at bin 1.01-1.02 were all significantly more susceptible than the ones carrying B73 allele(s) at the same region (Table 1.6), confirming the resistance effect of *qNLB1.02*_{B73}. Highly significant differences between TBBC3-42-10E-02 (the NIL with *qNLB1.02*_{Tx303} in a relatively cleaner background) and B73 were observed across all the disease components under investigation. The B73 allele(s) at *qNLB1.02* delayed lesion formation by 1.6 days ($P = 0.004$), inhibited lesion expansion by 0.84 mm per day ($P < 0.0001$), and reduced DLA 17-40% through the season ($P < 0.0001$). Overall, the relative allele effects detected in the NILs were much greater than in the segregating populations, and the resistance of *qNLB1.02*_{B73} was more effective in the field than in the greenhouse.

In addition to the major QTL at bin 1.02, there are likely other minor NLB QTL segregating in the NIL set derived from the B73 x TBBC3-42 cross. Minor effects, presumably contributed by introgression(s) (unidentified linked or unlinked loci), were inferred by comparing TBBC3-6F-02 and TBBC3-12F-01, which are genetically identical for the markers used in this study. Regardless of their similar genomic composition, TBBC3-6F-02 was consistently (but not significantly) more susceptible than TBBC3-12F-01 for disease components targeting earlier disease development. After disease accumulation through the season, significant background effects were observable in DLA3 and DLA4 (7-10%, $P < 0.003$) and AUDPC ($P = 0.015$). Bins 5.01-5.02, 7.01, 8.02, 8.03-8.05 were also analyzed, but none of the loci were found significantly affecting disease in the individual trait-locus analysis, and none of them

were found to be associated with resistance by comparing NILs and B73. Bins 7.01 and 8.02 were determined not effective for resistance, based on the non-significant difference observed among three NILs – TBBC3-42-05H-04, TBBC3-42-05H-04, and TBBC3-42-09E-01. There was weak evidence of NLB QTL at bins 5.01-5.02 and 8.03-8.05. The Tx303 allele(s) at bin 5.01-5.02 may be associated with resistance based on the significant difference observed between TBBC3-06F-02 and TBBC3-10E-02 for DLA1, DLA2 and AUDPC. Likewise, B73 allele(s) at bin 8.03-8.05 might be associated with resistance because TBBC3-42-05H-01 was significantly more susceptible than B73 for DLA3, DLA4 and AUDPC. But since the QTL effects detected at bins 5.02 and 8.03-8.05 were relatively minor, and the favorable alleles were not consistent with the results of linkage analysis, the possibility that the phenotypic difference was simply caused by background introgression(s) cannot be neglected. To determine whether bins 5.02 and 8.03-8.05 condition NLB resistance, the potential background QTL would need to be located by testing the NILs with more markers across the genome.

5. Characterization of *qNLB1.02*_{B73} and *qNLB1.06*_{Tx303} using derived NILs

Microscopic investigation on the pathogenesis of S. turcica. Trypan blue staining and KOH-aniline blue fluorescence microscopy techniques were used for histological examination of NLB development *in planta*. To understand typical resistant and susceptible interaction patterns in the NLB pathosystem, preliminary microscopic analysis was conducted on the following maize lines challenged with *S. turcica*: B73, Tx303, TBBC3-38, TBBC3-42, the resistant maize inbred line CML52 and CML103, and a susceptible recombinant inbred line IBM262. As illustrated in Fig. 1.3 and Fig. 1.4, pathogenesis was successfully visualized and can be summarized as follows. After landing and attaching on the leaf surface of a susceptible maize plant, the conidium of

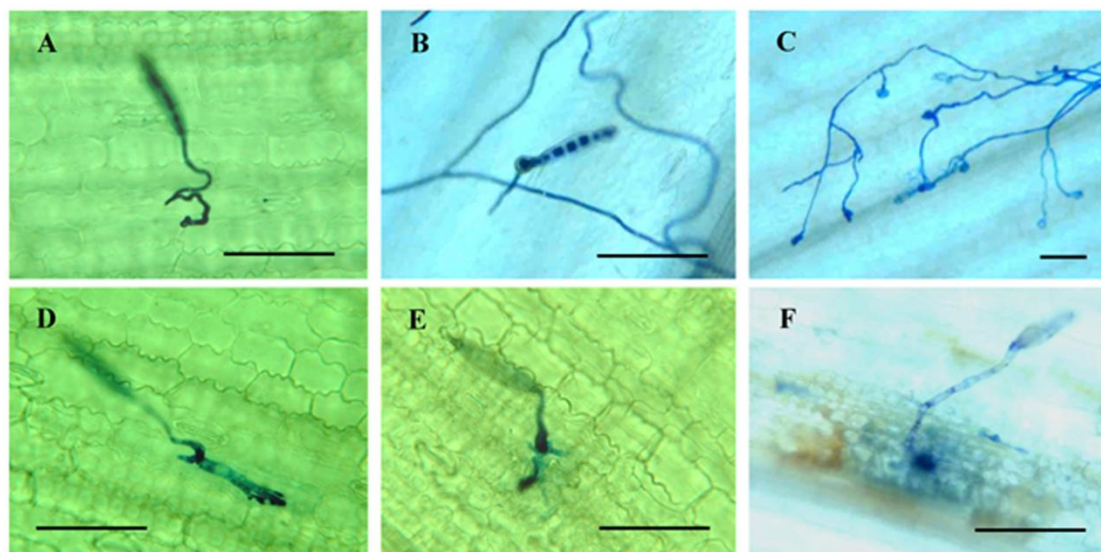
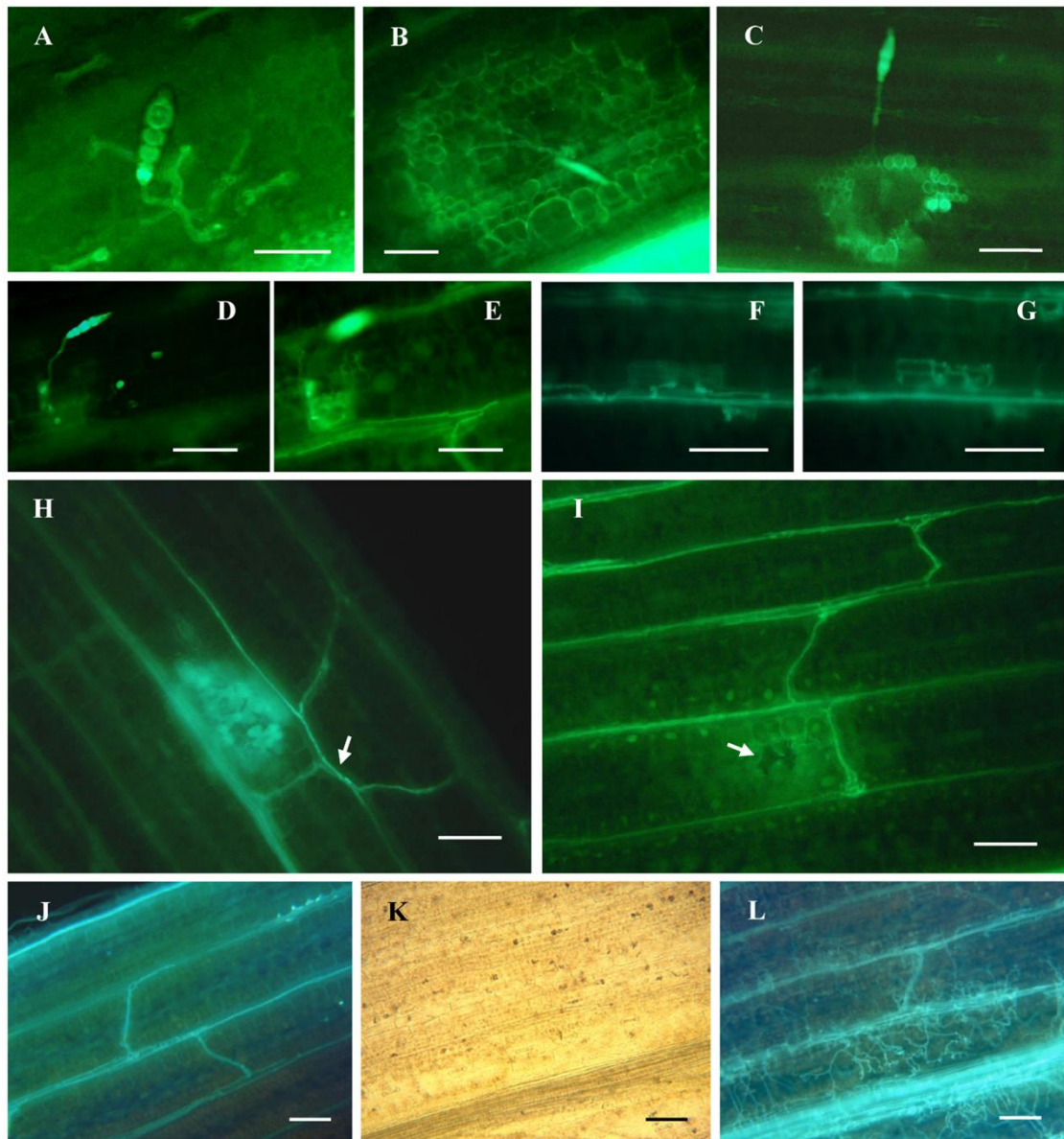


Figure 1.3 Light micrographs of the infection and early colonization of *S. turcica* in corn leaves. Samples were stained with trypan blue. (A) A conidium germinated and formed an appressorium. (B) The penetration peg from the appressorium punched through the cuticle and epidermal cell wall. (C) The conidium could continue to produce new germ tubes and appressoria until exhausting its reserves or ultimately gaining entry into a plant cell. This phenomenon was occasionally observed on a resistant inbred line CML52. (D) The subcuticular palm-shaped structure developed before hyphae infecting into the epidermal cell. (E and F) Cytoplasmic depletion of the conidium was seen after the infection process. Infective hyphae spread from primary infected cell to surrounding area, causing host cell death. (Infected leaf samples of A, D and E: CML52, 2dpi; B: B73, 2 dpi; C: CML52, 3 dpi; F: IBM262, 5 dpi) (Scale bars, 100 μ m)

Figure 1.4 Fluorescence micrographs of the pathogenesis of *S. turcica* in corn leaves. Samples were treated with KOH-aniline blue. (A) A germinated conidium on leaf surface. (B) Infective hyphae grew into contact with mesophyll cells. (C and H) Defense responses induced in the mesophyll cells and vascular bundles around the infection site. Bright fluorescing area is caused by callose deposition and the accumulation of autofluorescent phenolic compounds. The fluorescing vascular bundles (arrow) possibly due to the lignification can be differentiated from the hyphae growing in the vasculature, by the lack of distinguished hyphal coils. (D and E) Infective hyphae grew towards the vascular bundle and invaded into it. The focus levels of D and E were on leaf surface and vasculature, respectively. (F and G) Hyphae grew out the vascular vessel to colonize the neighboring bundle sheath cells. F and G were on different focus levels. (I) Hyphae successfully spread out through the vascular system. The weak fluorescence and collapsed cells surrounding the infection site (arrow) are typical symptoms of the compatible interaction. (J) Movement of infective hyphae through vascular bundles and cross veins. Leaf tissue remained non-wilted at this stage. (K and L) Colonization of necrotic hyphae in the wilted lesion. Vascular bundles were plugged with aggressively growing and extending hyphae. Hyphae branched out from the colonized vasculature to the rest of the leaf. K and L were viewed in the light- and fluorescent-field, respectively. (Infected leaf samples of A: Tx303, 4 dpi; B: CML52, 4 dpi; C: B73, 4 dpi; D and E: TBBC3-42, 4dpi; F and G: CML103, 7 dpi; H: B73, 4 dpi; I to L: B73, 10 dpi) (Scale bars, 100 μ m)



S. turcica germinates. It forms an appressorium, which produces a penetration peg that punches through the cell wall and into the epidermal cell. From this cell, infective hyphae are produced, grow towards the vascular bundle, enter the vasculature and ramify within it. Aggressive hyphal growth and extension in the vascular bundles can be seen. The hyphae can grow for several days inside the vascular bundles without causing visible symptoms (incubation period was approximately seven days on susceptible maize lines). After extensive growth within the vascular veins, hyphae grow out to colonize the neighboring bundle sheath cells and then branch out to colonize the rest of leaves. This is consistent with several previous studies that report a similar series of events during pathogenesis of *S. turcica* (Hilu and Hooker 1965; Jennings and Ullstrup 1957; Knox-Davies 1974). The limited damage *S. turcica* causes to host tissues at early phase of pathogenesis and the extended incubation period suggests the possibility that this is a hemibiotrophic interaction.

On maize genotypes carrying different QTL for resistance, differential phenotypes were observed for the timing and extent of certain steps in the processes of infection and colonization. To document these differences, a series of microscopic parameters was chosen for characterizing NLB QTL efficacy at different stages during pathogenesis. The microscopic disease components used in the study included: the number of appressoria, infection efficiency, vascular invasion efficiency, size of strong fluorescent area surrounding the infection site, and the appearance of necrotic or fortified vascular bundles. The microscopic evaluations on B73, Tx303 and the derived NILs were performed in controlled greenhouse condition as well as in the field.

a) QTL effect on the infection of *S. turcica*

Infection efficiency was examined by trypan blue and KOH-aniline blue

staining, followed by scoring the number of conidia that had successfully penetrated the epidermal cell walls. The infection efficiency was defined as the ratio of successful infection sites over the total number of germinated conidia. Non-germinated conidia were excluded because of the difficulty of determining whether a non-germinated conidium was viable, given the fact that a small portion of damaged conidia is always present in the spore suspension. In both the greenhouse and field, lower infection efficiency was consistently observed on the NIL with *qNLB1.06_{Tx303}* at earlier time points examined, as well as on Tx303 at any time points examined (Fig. 1.5A), compared with B73. In the greenhouse, *qNLB1.06_{Tx303}* reduced infection efficiency by ~14 % at 2 dpi and by ~24% at 4 dpi ($P < 0.0001$). Similarly, in the field, infection efficiency was reduced by ~12 % at 3 dpi ($P < 0.0001$) relative to B73. The more resistant parental genotype Tx303 showed a stronger effect on reducing the infection of *S. turcica* in the field (decreased by ~19 % relative to B73, $P < 0.0001$) than in the greenhouse (decreased by 6.5-12.5 % relative to B73, $P < 0.05$). No significant effect on pathogen penetration was seen from *qNLB1.02_{Tx303}*.

The incidence of multiple appressoria per germinated conidium was used as another indicator for resistance to fungal penetration. It was observed that when a germinated conidium failed to penetrate the epidermis, the conidium would continue to produce new germ tubes and appressoria until exhausting its reserves or ultimately gaining entry into a plant cell (Fig. 1.5B). Despite the evident effect of *qNLB1.06_{Tx303}* on reducing the penetration of *S. turcica*, no difference was detected on the formation of multiple appressoria among B73, Tx303, and the derived NILs.

b) QTL effect on the vascular invasion of *S. turcica*

Intracellular hyphal growth from the initially infected epidermal cell to surrounding mesophyll cells, and the subsequent invasion into the vascular bundle, were investigated by aniline blue – KOH fluorescence microscopy. Vascular invasion efficiency was defined as the incidence of hyphal growth into the vasculature per infection site. Differences in vascular invasion efficiency were observed between B73 and the other genotypes (Fig. 1.5C). The NIL with *qNLB1.02_{Tx303}* had greater vascular invasion efficiency, indicating the superiority of the B73 allele(s) at this locus. Replacement of the B73 allele with Tx303 allele at *qNLB1.02* (in lines carrying an introgression from Tx303 at this locus) led to 15-24 % of increase in the incidence of vascular invasion in both greenhouse and field ($P < 0.0001$). The *qNLB1.06_{Tx303}* allele(s) was not effective for inhibiting hyphal growth into the vasculature. Under greenhouse conditions, the NIL with *qNLB1.06_{Tx303}* was observed to have a 7-19% higher susceptibility for vascular invasion ($P < 0.0001$), which is a further indication that this QTL functions at the stage of penetration but not at later stages of pathogenesis. The susceptibility to vascular invasion was also seen in Tx303. In the greenhouse, although with marginal significance levels, higher vascular invasion efficiencies were observed in Tx303 than in B73. In the field, the invasion efficiency in Tx303 was ~22 % greater than in B73 ($P < 0.0001$).

c) QTL effects on the accumulation of defense compounds around the infection site

Infected tissues stained with aniline blue were also used for revealing defense responses induced upon pathogen challenge. The fluorescence emitted from the cells surrounding the infection site is presumably attributed to callose deposition (Gonzalez et al. 2006; Johnston et al. 2006) and the accumulation of

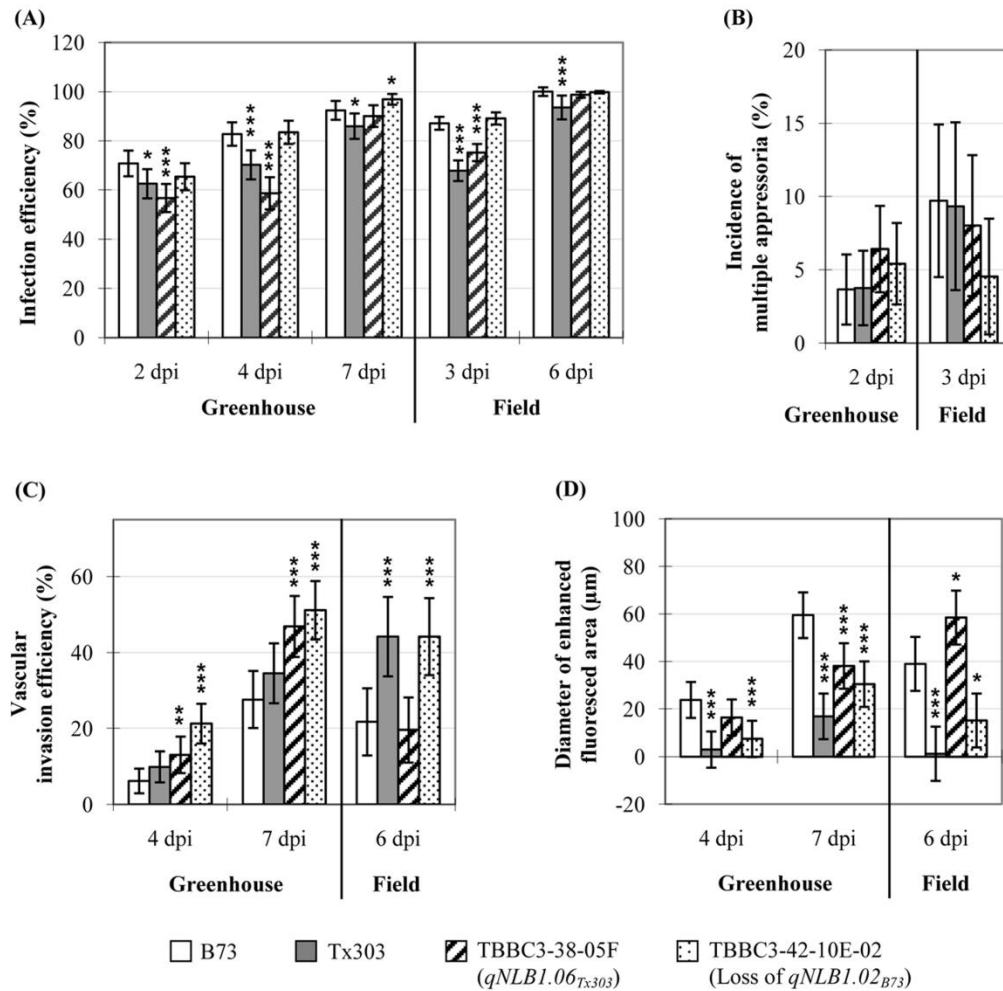


Figure 1.5 Investigation of QTL effects on microscopic disease components including (A) infection efficiency, (B) incidence of multiple appressoria, (C) vascular invasion efficiency, and (D) size of strong fluoresced area surrounding the infection site, in controlled greenhouse condition and field. Infected leaf samples were collected from maize genotypes B73 (open), Tx303 (gray), TBBC3-38-05F (hatched; the NIL carrying *qNLB1.06*_{Tx303}) and TBBC3-42-10E-02 (dotted; the NIL carrying *qNLB1.02*_{Tx303}, which is essentially “loss of *qNLB1.02*_{B73}”). Samples collected 2 days post inoculation (dpi) from greenhouse and 3 dpi from field were stained with trypan blue, while the samples collected 4 dpi and 7 dpi from greenhouse and 6 dpi from field were treated with KOH-aniline blue fluorescence technique. Differences between least squares means of different genotypes relative to B73 were determined by two-tailed Student’s t test (significance level: * 0.01 < *P* < 0.05; ** 0.001 < *P* < 0.01; *** *P* < 0.001). In the graphs of A to C, the proportion data were arcsine transformed for statistical analysis, and the corresponding least squares means and 95% confidence intervals were back transformed to original scale before plotting. The confidence intervals are bigger than significance levels due to asymmetry resulting from back transformation.

autofluorescent phenolic compounds (Bennett et al. 1996; Ficke et al. 2002; Soylu 2006). Callose and phenolics are antimicrobial compounds that help restrict the establishment and spread of *S. turcica* in the leaf. In susceptible genotypes, weak fluorescence and collapsed cells were generally observed around the infected sites (Fig. 1.4I), suggesting the lack of induced defense responses. The more resistant genotypes showed enhanced fluorescence from localized cell regions (Fig. 1.4C and 4H).

To assess the degree of defense response around the primary infected cell, the diameter of brightly fluorescing area was measured under a fluorescence microscope with a micrometer (Fig. 1.5D). The average diameter of the fluorescing area in the NIL with *qNLB1.02_{Tx303}* was consistently smaller than that seen for B73 ($P < 0.0001$ in greenhouse, $P = 0.02$ in the field), indicating that the B73 allele(s) at *qNLB1.02* contribute to the the regulation, production or accumulation of callose, lignin or other phenolic compounds. Interestingly, compared to B73, the fluorescing area in the NIL with *qNLB1.06_{Tx303}* was slightly larger ($P = 0.052$) in the field, but was significantly smaller ($P < 0.0001$) at 7 dpi in the greenhouse, suggesting that the phenotype conferred by *qNLB1.06_{Tx303}* is vulnerable to environmental influences.

Considering the potential role of vascular resistance in the NLB pathosystem, the incidence of bright fluorescing vascular bundles (likely caused by lignification) was also evaluated. Although this response was pronounced in CML52 (Fig. 1.4H), this type of resistance was rarely observed in B73, Tx303 and the derived NILs. The induced defense reaction around the infection site was fairly weak at all times examined in either greenhouse or field conditions. It was thus not considered a relevant parameter for testing the effect of *qNLB1.06_{Tx303}* and *qNLB1.02_{B73}*.

d) QTL effect on mycelial growth of *S. turcica* in planta

DNA-based real-time PCR was developed to precisely quantify mycelial growth of *S. turcica* in maize leaves. The *R*-squares for the two standard curves based on B73 and Tx303 respectively were both higher than 0.99 (Fig. 1.6A), indicating the designed ITS primer pair had good sensitivity and specificity for reliably amplifying fungal DNA. To assess quantitative PCR technique as a tool for analyzing the NLB pathosystem, several time-course experiments were performed on seven maize genotypes with a wide range of differential levels of NLB resistance (C. Chung, unpublished). Preliminary results showed that the levels of fungal DNA ratios determined by qPCR approximately conformed to the performance of resistance in the field. For maize genotypes with low to intermediate levels of resistance, fungal DNA was detected as early as three days after inoculation. The detectable fungal DNA ratios increased over time until the end of the incubation period (when visible lesions formed), and thereafter decreased. This reduction in pathogen DNA was unexpected, and apparently corresponded to a loss of DNA integrity. DNA samples extracted from tissues showing lesions were usually brownish and of poor quality. The decrease in detectable fungal DNA ratio in highly diseased leaves is contradictory to our microscopic observation of abundant mycelial growth in NLB lesions. It is likely that in tissues actively developing lesions, more DNA degradative enzymes involving in cell death are present and can degrade fungal DNA during the extraction. To better differentiate resistant and susceptible responses, it is critical to apply qPCR on infected tissues taken prior to the appearance of necrotic lesions on the most susceptible genotype in an experimental set.

Infected leaves from B73, Tx303, and two derived NILs were collected 9 days after inoculation (according to the IP of the susceptible NIL), and measured for

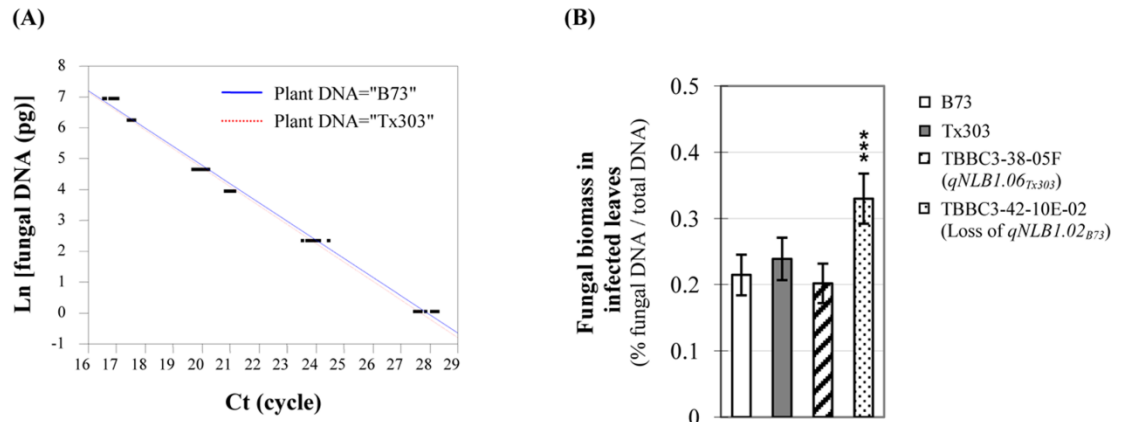


Figure 1.6 Quantifying QTL effect on mycelial growth of *S. turcica* in planta using DNA-based real time PCR. (A) Two standard curves were constructed by mixing a series of *S. turcica* DNA and 50 ng of maize DNA from non-inoculated B73 and Tx303 plants, respectively. With B73 DNA, $\text{Ln} [\text{fungal DNA}] = 16.912 - 0.606 \cdot \text{Ct}$ ($R^2 = 0.99$); with Tx303 DNA, $\text{Ln} [\text{fungal DNA}] = 16.906 - 0.610 \cdot \text{Ct}$ ($R^2 = 0.99$). Ln: natural logarithm. Ct: threshold cycle, the number of PCR amplification cycle at which the exponential increase of the product is detected. (B) Measurement of *S. turcica* DNA ratio in infected leaves collected 9 dpi from maize genotypes B73 (open), Tx303 (gray), TBBC3-38-05F (hatched; the NIL carrying $qNLB1.06_{Tx303}$) and TBBC3-42-10E-02 (dotted; the NIL carrying $qNLB1.02_{Tx303}$, which is essentially “loss of $qNLB1.02_{B73}$ ”). Differences between least squares means of different genotypes relative to B73 were determined by two-tailed Student’s t test (significance level: * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$). The proportion data were arcsine transformed for statistical analysis, and the corresponding least squares means and 95% confidence intervals were back transformed to original scale before plotting. The confidence intervals are bigger than significance levels due to asymmetry resulting from back transformation.

their fungal DNA content using qPCR. As shown in Fig. 1.6B, neither Tx303 nor *qNLB1.06*_{Tx303} showed significant effect on reducing the growth of *S. turcica* in leaves. However, the fungal biomass ratio (% of fungal DNA in the infected leaf) in the NIL with *qNLB1.02*_{Tx303} was 24 % higher than in B73 ($P < 0.0001$), indicating that the *in planta* development of *S. turcica* is more extensive on plants without *qNLB1.02*_{B73}.

6. Preliminary characterization of potential QTL effects for multiple disease resistance using derived NILs

Significant differences were observed between Tx303 and B73 for resistance to Stewart's wilt, anthracnose stalk rot (ASR), common smut and common rust. In the field, Tx303 was more resistant to Stewart's wilt and ASR, while B73 was more resistant to common smut and common rust. To investigate the resistance spectrum of *qNLB1.02* and *qNLB1.06*, several NILs derived from B73 x TBBC3-38, B73 x TBBC3-39 and B73 x TBBC3-42 were evaluated at Aurora NY for ASR and smut in 2007 and 2008, and for Stewart's wilt and rust in 2008 and 2009 (Table 1.5 and 1.6). The choice of NILs was constrained by the availability of seeds. Plant materials used in 2007 were BC₄F₃ NILs, and the plants used in 2008 and 2009 were BC₄F₄ NILs. For ASR and smut, the 2008 data resulting from the NILs with cleaner backgrounds are shown to represent the overall outcome (similar results observed in 2007 and 2008). The same analytical approaches used for declaring NLB QTL in TBBC3 population and derived NIL sets were used to determine the effects of *qNLB1.02* and *qNLB1.06* for other diseases.

The evidence suggested that both *qNLB1.06*_{Tx303} and *qNLB1.02*_{B73} were effective for resistance to Stewart's wilt. As shown in Table 1.5, all the NILs carrying Tx303 introgression at bin 1.06 were significantly more resistant than B73. Based on

comparing TBBC3-39-19E to B73, *qNLB1.06*_{Tx303} was inferred to be effective for reducing lesions of Stewart's wilt on inoculated leaves by as much as 39% ($P < 0.0001$). Likewise, in Table 1.6, lines with Tx303 alleles at bin 1.01-1.02 were significantly more susceptible than B73. The effect of *qNLB1.02*_{B73} on Stewart's wilt was estimated as reducing 19% of PrimDLA based on a comparison of TBBC3-42-10E-02 and B73 ($P < 0.0001$). Significant differences were also observed among the lines fixed (homozygous for Tx303 or B73 alleles) at bins 1.01-1.02, indicating that there are QTL other than *qNLB1.02*_{B73} segregating in the NIL set. However, none of the rest of the introgressed regions were unambiguously associated with resistance. For example, Tx303 allele(s) at bin 8.03-8.05 seemed to be associated with resistance, based on the significantly greater resistance in TBBC3-42-05H-01 relative to B73. But this presumably QTL was not effective in TBBC3-05H-04 and TBBC3-09E-01, suggesting that the true QTL position was not closely linked with *umc1130*, or that some antagonistic effects were associated with bin 8.02 and/or unidentified introgression(s).

The QTL designated *qNLB1.06* was ineffective for resistance to ASR, smut and rust. No phenotypic difference in the development of rust and smut galls was detected among B73, TBBC3-38, TBBC3-39 and their derived NILs. Significant difference in discolored internode area was observed between TBBC3-38 and B73 (~32%, $P = 0.001$), but the variation was not associated with *qNLB1.06*, as the NIL carrying a single introgression at bin 1.06 (TBBC3-39-19E) showed the same resistance level as B73 (Table 1.5).

The QTL designated *qNLB1.02* was ineffective for resistance to ASR and common smut, but potentially effective for resistance to common rust (Table 1.6). For ASR, some phenotypic difference was found among B73, TBBC3-42 and derived NILs in the 2007 and 2008 trials, but none of the identified introgressed regions were

associated with resistance. B73 was more resistant than Tx303 for common smut (differed by ~1.5 units of a 10-unit scale in stalk gall severity, $P < 0.0001$; ~2.5 units of a 10-unit scale in ear gall severity, $P < 0.0001$) and common rust (differed by 1-1.5 units of a 10-unit scale in severity through the season, $P < 0.036$; ~24.8 scale-day in AUDPC, $P = 0.027$). For common smut, there were almost no ear galls or stalk galls observed on B73, TBBC3-42 and TBBC3-42-10E-04, suggesting that introgressed segments in TBBC3-42 did not affect smut development. (More ear galls were scored on TBBC3-42-10E-04 in the 2008 trial, but the significance was marginal ($P = 0.04$), and the finding was not consistent with the 2007 result.) For common rust, except for TBBC3-42-06F-2, lines with Tx303 alleles at bin 1.01-1.02 were consistently more susceptible than B73 for all three severity ratings (differed by 1.1-3.1 scales in severity, $P < 0.025$) and AUDPC (differed by about 43-53 scale-day, $P < 0.001$). The result indicated the possibility of rust QTL at bins 1.01-1.02 and/or unidentified introgressed regions in TBBC3-42.

DISCUSSION

1. Identification of NLB QTL using TBBC3 introgression lines

Two QTL for resistance to NLB, $qNLB1.02_{B73}$ and $qNLB1.06_{Tx303}$, were successfully identified, validated and characterized using a population of introgression lines and derived NILs. We applied a stepwise strategy that allowed phenotyping of informative NILs over a series of generations. A special field design, in which each row of CSSL/NIL arranged next to a row of B73, allowed accurate visual comparisons and a relatively uniform epidemic condition across the field. We detected and sequentially validated QTL using multiple disease components, in a full set of 82 lines, a subset of 15 selected lines, 5 selected BC₄F₂ populations, and 3 sets of derived

BC₄F₃/ BC₄F₄ NILs. Our primary hypothesis that individual QTL affect distinct stage(s) of NLB development was ultimately confirmed using two selected NILs (compared to B73) in repeated greenhouse and field trials, with a panel of conventional and novel components. Our results revealed that *qNLB1.06*_{Tx303} and *qNLB1.02*_{B73} are mainly effective against the infection and colonization of *S. turcica*, respectively. We found that the QTL were both effective in both juvenile and adult plants, and that both chromosomal segments were associated with resistance to more than one disease.

The mechanism underlying resistance conferred by *qNLB1.02*_{B73} and *qNLB1.06*_{Tx303} is unlikely to be the same as the mechanism underlying qualitative resistance conferred by currently known major genes. Qualitative resistance to NLB is generally characterized by chlorotic-necrotic lesions (Hooker 1963, 1977, 1981; Ogliari et al. 2005; Ogliari et al. 2007), chlorotic halo lesions (Carson 1995b), or extremely prolonged IP (Gevers 1975). Considerable levels of resistance were seen for *qNLB1.02*_{B73} and *qNLB1.06*_{Tx303} in the two field sites, without observing distinct lesion type or much greater IP. Moreover, although co-localized NLB QTL at bins 1.02 and 1.06 have been reported in the populations of B52 x Mo17 (Freymark et al. 1993) and D32 x D145 (Welz et al. 1999b), respectively, no major genes have been mapped to these chromosomal regions.

While the detailed characterization was focused on two QTL, bins 1.11, 4.03, 5.04, and 7.03 were found to be associated with NLB resistance, based on the analysis of the full set and a subset of TBBC3 lines. The original TBBC3 pop was genotyped with 130 informative markers spaced throughout the genome (Szalma et al. 2007). Although this represents very good coverage of the genome, it is likely that some introgressions, located entirely within genotyped intervals, remain unidentified. More markers would have to be applied across the genome to reveal the background

introgressions. In fact, *qNLB1.06_{Tx303}* was not detected until the analysis of 68 additional SSRs targeting chromosomal regions that were not well covered by the original marker set.

Disease resistance is a relative term. Most genotypes express some degree of quantitative resistance compared to extremely susceptible lines, so disease QTL can be identified even in genotypes considered to be susceptible. In most published disease QTL studies, QTL for resistance have been identified in the susceptible as well as the resistant parents [eg. Freymark *et al.* (1994); Brown *et al.* (2001)]. Nearly half of the TBBC3 lines showed greater susceptibility than B73. The overall susceptibility of the CSSL population suggested that Tx303 carries fewer QTL, and B73 carries many minor QTL. In addition to QTL identified in the present study, more loci conditioning disease resistance can be found in B73. These disease QTL may be involved in plant defense in a manner other than typical R-gene resistance, since no qualitative resistance has been reported in B73. Uncovering the presumably favorable B73 alleles would be valuable for practical reasons, as the elite inbred line B73 has been commonly used in many breeding programs because of its agronomic performance. In reflecting on QTL not detected here, it should be acknowledged that the use of CSSL/NIL-based analysis is not an efficient approach to identifying epistatic effects between QTL. The effects of single Tx303 introgressions could have been underestimated due to lack of epistatic interactions in the B73 background.

2. Conventional and newly-developed components of resistance targeting different stages of NLB development

QTL effects were analyzed using five conventional macroscopic disease parameters and four microscopic parameters. The macroscopic components were IP, LE, PrimDLA, DLA/disease severity, and AUDPC, and each was evaluated in various

environments. The components IP and LE reflect different phases of disease development. IP quantifies the time to appearance of wilted lesions, which reflects the speed of xylem plugging due to extensive hyphal growth in the veins. LE quantifies the rate of expansion of wilted and necrotic lesions, which reflects the speed of destructive hyphal growth in the leaves. Ratings for DLA, disease severity and AUDPC, on the other hand, involve visual quantification of overall disease progress on the entire plant. In our greenhouse and field trials, IP showed a better correlation than LE with DLA, disease severity and AUDPC. This indicates that IP is a more discriminating parameter than LE for measuring NLB severity. This confirms previous reports that IP² is a convenient target trait in selection and breeding for resistance to NLB (Brewster et al. 1992; Carson 1995a, 2006; Schechert et al. 1999; Smith and Kinsey 1993; Welz et al. 1999a; Welz et al. 1999b). In a recurrent selection study for NLB resistance, selection for prolonged IP/LP, resulted in ~7.5% more gain in reducing AUDPC per selection cycle compared to selection for lesion length (Carson 2006).

QTL effects were also microscopically investigated at the pre-penetration, infection, and colonization phases. In most fungal pathosystems, pre-penetration resistance is associated with specialized physical and chemical features of plant surface which help reduce the incidence of landing, adhesion, germination, appressorium formation and penetration of pathogenic fungi (Tucker and Talbot 2001). Post-penetration resistance to microbial attack, on the other hand, is characterized by programmed necrosis, along with the induction of callose, lignin, phenolic compounds and other pathogenesis-related proteins around the primary-infected cell. Four microscopic components, including the incidence of multiple

² In some publications, the term “latent period” has been used to score the time to appearance of necrotic lesions, rather than the time to sporulation. Latent period in those reports is thus essentially equivalent to IP in our study.

appressoria, infection efficiency, accumulation of defense materials surrounding the infection site, and vascular invasion efficiency, were developed to complement the conventional macroscopic components. Modified methods of trypan blue staining (Knox-Davies 1974; Vélez 2005) and KOH-aniline blue fluorescence technique (Hood and Shew 1996) were proven to be applicable in understanding the degree and timing of allelic contribution to quantitative resistance to NLB. Previous studies utilizing sectioning and electron microscopy have revealed that xylem plugging is a key stage for the formation of NLB lesions (Jennings and Ullstrup, 1957). Our microscopic examination using KOH-aniline blue technique confirmed the finding. With KOH-aniline blue treatment, mycelium could be easily distinguished from plant tissues by the intensity of fluorescence and the unique hyphal structure (Hood and Shew 1996). Hyphae inside the epidermal cells, mesophyll cells, bundle sheath cells or vascular bundles could be readily visualized at 40x. This technique also allowed us to view the *in planta* colonization of *S. turcica* from a different angle. In all the maize genotypes examined, the mycelium appeared to grow preferentially towards vascular bundle, initially invading the vasculature, then extending through the xylem vessel, and eventually, aggressively growing out to the neighboring mesophyll tissues. To our knowledge, the mechanisms of the post-infection directional growth of vascular fungal pathogens remain to be elucidated.

Quantitative PCR (qPCR) was employed for the first time for the measurement of *in planta* biomass of *S. turcica*. Although it has been widely applied in other pathosystems, qPCR can only be used for NLB quantification at early stages of pathogenesis. Due to the nature of NLB development, the accuracy and differentiating power are highly dependent on uniform inoculation and precise sampling at the early stages of pathogenesis, so the method is best suited to work conducted under controlled conditions. The qPCR technique only provided meaningful results with the

use of infected tissues prior to the formation of lesions. The biggest constraint of this technique comes from its not being able to measure the destructive mycelial growth in the blighted leaf areas, possibly due to the poor quality of DNA extracted from necrotic tissues. The cost of qPCR reagents and the required workload make it unfeasible for regular phenotypic screening. However, qPCR serves as a good tool for quantifying the degree of earlier mycelial colonization of *S. turcica* in maize leaves.

3. Hypothesis #1: individual QTL affect distinct stages of disease development

Detailed macroscopic and microscopic evaluations revealed the distinct features of *qNLB1.06_{Tx303}* and *qNLB1.02_{B73}* in NLB development. *qNLB1.06_{Tx303}* conditions resistance mainly against the penetration of *S. turcica*. The anti-penetration effect was observed at earlier but not later time points examined, indicating that *qNLB1.06_{Tx303}* acts in a quantitative manner, which delays rather than prevents the occurrence of infection. In contrast, *qNLB1.02_{B73}* appears to conditions resistance not to infection, but rather by enhancing the induction of defense reactions surrounding infection sites, as well as by inhibiting hyphal growth into the vascular bundle, and the subsequent necrotrophic colonization in the leaves. It is difficult to infer whether the resistance is associated with defense reactions in mesophyll, parenchyma, bundle sheath and/or other cells.

Delaying the invasion and extension of *S. turcica* in the vascular system was shown to be critical for quantitative resistance to NLB. This is consistent with previous microscopic analyses (Hilu and Hooker 1965; Jennings and Ullstrup 1957) suggested that xylem invasion/plugging is an important pathogenetic phase in NLB progress. Polygenic resistance (in an inbred line CI90A) has been associated with reduced hyphal spread into the xylem (Hilu and Hooker 1964). The significance of protecting the vasculature from hyphal invasion is supported by the evidence that

*qNLB1.02*_{B73} conferred a much greater overall resistance than *qNLB1.06*_{Tx303} in the field. Replacement of superior B73 allele with Tx303 allele at *qNLB1.02* led to 18-38 % higher DLA relative to B73, while replacement of the inferior B73 allele with Tx303 allele at *qNLB1.06* only decreased DLA by 2-10 %, relative to B73. Similarly, the superior allele at *qNLB1.02* reduced AUDPC by 114%, while the superior allele at *qNLB1.06* reduced AUDPC only by 29% (Table 1.5 and 1.6). Incorporating a QTL effective in slowing down the invasion of the vasculature would thus considerably increase overall resistance.

In the donor line Tx303, it is likely that preventing the attachment of conidia and the subsequent infection plays a role in quantitative resistance to NLB. Although not actually quantified, the number of spores per leaf segment of Tx303 was obviously much less than the spore numbers on B73 or the NILs carrying *qNLB1.06*_{Tx303} and *qNLB1.02*_{B73}. Less infection efficiency of *S. turcica* was also found in Tx303 than in B73. Interestingly, Tx303 showed a greater susceptibility than B73 for the parameters of vascular invasion efficiency, enhanced fluoresced area, and lesion expansion. Despite the lack of resistance in delaying vascular invasion and extension of *S. turcica* in Tx303, its higher effectiveness in reducing spore attachment and infection may contribute to the overall moderate resistance to NLB.

4. Hypothesis #2: the effectiveness of disease QTL is affected by plant maturity

A number of maize diseases caused by hemibiotrophic fungi (eg. gray leaf spot, anthracnose leaf blight, anthracnose stalk rot) and necrotrophic fungi (eg. southern leaf blight) are known to progress more rapidly on the plants after anthesis (Bubeck et al. 1993; Carson 1999; Keller and Bergstrom 1988; Leonard and Thompson 1976). In maize, a significant correlation has been detected between disease resistance and flowering time in a panel of 253 diverse lines (R. Wisser, J. Kolkman, P. Balint-Kurti,

and R. Nelson, pers. comm.). The fact that flowering time may account for a considerable proportion of resistance variation leads to the hypotheses that the effects of disease QTL reflect indirect expression of flowering time QTL, and/or that the effects of disease QTL are modulated differently at varied plant developmental stages. In fact, several QTL identified in biparental populations are associated with both NLB resistance and flowering time (J. Poland, pers. comm.).

The association between NLB QTL and plant maturity was investigated in the subset of 15 selected TBBC3 lines. To separate resistance effects from maturity effects, plants in two different fields were inoculated at juvenile and adult stage (two weeks before tasselling) respectively, and evaluated for NLB resistance and flowering time. Generally consistent expression of resistance or susceptibility was observed at the two stages, implying that the effectiveness of these QTL was not altered substantially by plant maturity. While several of the introgression lines (10 out of the 15 TBBC3 lines with differential resistance relative to B73) showed significantly different flowering times relative to B73, the interactions between NLB QTL and flowering time QTL were relatively minor. For the *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}*, the analysis of advanced NIL sets further provided strong evidence of their independence from flowering time. This agrees with a previous report of flowering-time QTL in TBBC3 introgression lines (Szalma et al. 2007): bins 1.01-1.02 and 1.06 were not among the QTL affecting days to anthesis, days to silking, or anthesis-silking interval.

5. Hypothesis #3: the effectiveness of QTL is affected by environmental conditions

A major limitation for QTL applications is the lack of consistency of QTL effects across environments. The inconsistent detection of QTL has been associated with

experimental errors and differential gene expression affected by environmental factors (Sofi and Rather 2007). In view of the widely reported inconsistency, the present study revealed that the introgressions/QTL with relatively larger effects (carried in seven introgression lines: TBBC3-38, TBBC3-39, TBBC3-42, TBBC3-36, TBBC3-21, TBBC3-30, and TBBC3-77) were consistent in their performance across field sites and years. Reliable expression of these QTL, including *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}*, suggests their applicability to resistance breeding.

Acceptable conformity was observed for the performance of the introgression lines in different years at each given field site. In repeated trials at each site, no lines showed significantly contrasting levels of resistance relative to B73. On the other hand, QTL by field site interactions were detected for a few introgression lines. In different years, TBBC3-14, TBBC3-19, and TBBC3-61 were consistently more resistant or more susceptible than B73 at only one field site or the other. This indicates potential modulation of some of the underlying NLB QTL in certain environments.

Disease resistance can expressed differentially, not only in different field environments but also under field versus greenhouse conditions [eg. Bubeck *et al.* (1993); Trognitz *et al.* (2001); Dinh *et al.* (2007)]. Differential efficacy of *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}* in controlled greenhouse and field conditions was evaluated using macroscopic and microscopic phenotypes. It should be noted that due to the different inoculation and sampling treatments necessarily employed in different environments, the comparison between the effectiveness of disease QTL in the greenhouse and field should be addressed based on relative observation rather than the absolute values. Our overall results suggest that the resistance of *qNLB1.02_{B73}* was stably expressed in the greenhouse and field, whereas *qNLB1.06_{Tx303}* conferred a higher level of resistance in the field than in the greenhouse. Macroscopically, the effects of *qNLB1.06_{Tx303}* on IP and PrimDLA in greenhouse-grown plants were

occasionally insignificant. Microscopically, the effect of *qNLB1.06_{Tx303}* on triggering defense response surrounding the initially infected cell was only detected in the field. Greenhouse-grown plants carrying *qNLB1.06_{Tx303}*, however, displayed an opposite effect (smaller fluorescent area), which suggests that this type of resistance is highly affected by environmental factors. The observation that *qNLB1.06_{Tx303}* increased the efficiency of *S. turcica* invasion into vasculature in the greenhouse but not field, may be an indirect effect of decreased accumulation of host defensive materials at the post-infection stage.

6. Hypothesis #4: QTL for resistance to NLB comprises genes or gene clusters involving in broad-spectrum resistance

The phenomenon of multiple disease resistance (MDR) has been inferred based on the detection of QTL clusters affecting different diseases (Welz and Geiger 2000; Wisser et al. 2006; Wisser et al. 2005), genetic correlations in populations (Mitchell-Olds et al. 1995), non-specific defense mechanisms [eg. SAR (Asai et al. 2002; Durrant and Dong 2004; Yang et al. 1997)], and genes [eg. *npr1* (Mou et al. 2003) and *mlo* (Consonni et al. 2006)]. Maize bin 1.02 and 1.06 have been previously associated with a number of disease QTL mapped in diverse maize populations (Wisser et al. 2006). Our evaluations of resistance to common rust, Stewart's wilt, anthracnose stalk rot, common smut, and common rust in sets of NILs suggest that both *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}* encompass gene(s) contributing nonspecific defense effects. *qNLB1.02_{B73}* was effective in decreasing NLB, common rust and Stewart's wilt, while *qNLB1.06_{Tx303}* was effective in decreasing NLB and Stewart's wilt. Nevertheless, due to the low resolution of QTL localization in this study, it is unclear whether the nonspecific resistance of *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}* is caused by pleiotropy or linkage, or whether their effects are monogenic or polygenic. Although the underlying

mechanisms are unknown, the feature of broad-spectrum resistance makes the two identified disease QTL appealing in practical applications.

It is worth noting that the NILs carrying *qNLB1.06_{Tx303}* showed remarkable resistance against Stewart's wilt, suggesting the involvement of major gene effect. In fact, a dominant major gene locus for Stewart's wilt, *Sw1*, has been mapped to bin 1.05-1.06 in inbred line Ki14 (Ming et al. 1999; Pataky et al. 2008). The co-localization of QTL for resistance to NLB and Stewart's wilt has also been observed in the NILs derived from the inbred line CML52 crossed to B73 (CML52 allele for resistance; details in Chapter 2).

CONCLUSION

Our research has led to successful identification of two reliably-expressed QTL that can potentially be utilized to protect maize from *S. turcica* in different environments. Map-based cloning will reveal more about the genes and mechanisms underlying the distinct features of *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}* in the pre-penetration, penetration and post-penetration phases of pathogenesis. Large mapping populations have been generated from the NILs and fine-mapping of *qNLB1.02_{B73}* and *qNLB1.06_{Tx303}* is in progress.

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CHAPTER 2

TARGETED DISCOVERY OF QUANTITATIVE TRAIT LOCI FOR RESISTANCE TO NORTHERN LEAF BLIGHT AND OTHER DISEASES IN MAIZE³

ABSTRACT

With the aim of capturing diverse alleles at a set of loci associated with disease resistance in maize, the heterogeneous inbred family (HIF) analysis (Tuinstra et al. 1997) was applied for targeted QTL mapping and near-isogenic line (NIL) development. Two tropical maize lines, CML52 and DK888, were chosen as donors of alleles based on their superior resistance to multiple diseases. Chromosomal regions (“bins”) associated with multiple disease resistance (MDR) were selected based on a consensus map of disease QTL in maize. For the target chromosomal regions, residual heterozygotes were identified among the available near-inbred lines of B73 x CML52 and S11 x DK888, respectively. For the 94 CML52-derived lines in 19 F₅ families, 64 simple sequence repeat (SSR) markers were used to target 38 bins. For the 46 DK888-derived lines in 17 F₆ families, 17 SSR markers were used to target 11 bins. We generated HIFs consisting of NILs segregating for the targeted loci but isogenic at > 96.9 % of the genome. Alleles at 30 target bins were evaluated in segregating HIFs for resistance to the primary disease of focus – northern leaf blight (NLB). To validate

³ Jesse Poland designed and managed the evaluation of the RILs for resistance to NLB and ASR, and conducted the statistical analyses for the RIL-based QTL mapping. Kristen Kump and Peter Balint-Kurti designed and managed the evaluation of the RILs for resistance to SLB. P. Balint-Kurti conducted phenotypic evaluation of the HIFs for resistance to SLB and GLS. Jacqueline Benson designed and managed the evaluation of the RILs and HIFs for resistance to GLS. Joy Longfellow and Ellie Walsh participated in phenotypic data collection.

candidate NLB QTL, and to test the hypothesis that the CML52 and DK888 alleles at MDR hotspot regions condition broad-spectrum resistance, sets of NILs were derived and evaluated for resistance to NLB and seven other important maize diseases, including southern leaf blight (SLB), gray leaf spot (GLS), anthracnose leaf blight (ALB), anthracnose stalk rot (ASR), common rust, common smut, and Stewart's wilt. Four NLB QTL, two ASR QTL, and one Stewart's wilt QTL were identified and validated using HIF-based approach. In parallel, a conventional QTL mapping study was conducted. A population of 196 recombinant inbred lines (RILs) derived from B73 x CML52 was evaluated for resistance to NLB, GLS, SLB and ASR. Six NLB QTL, four SLB QTL, and two ASR QTL were mapped. A high correspondence was found between the QTL identified using HIFs and RILs. Combining HIF- and RIL-based analyses, we discovered three disease nonspecific QTL, at which CML52 alleles were favorable. A QTL in bin 1.06 conferred resistance to NLB and Stewart's wilt; a QTL in bin 1.08 conferred resistance to NLB and SLB; and a QTL in 6.05 conferred resistance to NLB and ASR. The non-specific resistance may be attributed to pleiotropy or linkage. In spite of the lower power of QTL detection and some ambiguity associated with genetic background effect, HIF analysis proved to be an effective approach to NIL development and QTL characterization.

INTRODUCTION

Genetic architecture of disease resistance has become better understood, with increasing identification and mapping of R-genes, resistance gene analogs (RGAs), defense response gene homologs (DRHs) and loci conditioning quantitative disease resistance (quantitative trait loci for disease, or disease QTL) in a range of plant genomes. It has been widely recognized that R-genes, RGAs, DRHs and disease QTL

are not evenly distributed across the genome (Kanazin et al. 1996; Lopez et al. 2003; Wang et al. 2007a; Wang and Xiao 2002; Wisser et al. 2006; Wisser et al. 2005). Major genes and QTL for a given disease have frequently been co-localized to certain genomic regions [eg. *rhm* and QTL for southern leaf blight, and *Rp3* and QTL for common rust in maize (Wisser et al. 2006)], suggesting potentially overlapping features of qualitative and quantitative resistance. Different degrees of resistance may reflect allelic variants of identical gene(s) (Robertson 1989; Welz and Geiger 2000), or differential performance of resistance in various genetic backgrounds or environmental conditions. Insights into the spectrum of resistance conferred by a given locus have also been gained. Apparent clustering of QTL for different diseases have been commonly observed (Williams 2003; Wisser et al. 2006; Wisser et al. 2005), leading to the hypothesis that some chromosomal segments are associated with multiple disease resistance (MDR). Moreover, mapping of defense response gene homologs (DRHs) (Faris et al. 1999; Li et al. 1999; Ramalingam et al. 2003; Wang et al. 2007a) has revealed their co-localization with some disease QTL in plants. This implies that part of disease QTL may be controlled by DR genes (conserved defense machinery) (Faris et al. 1999), and therefore may contribute nonspecific resistance.

Quantitative disease resistance is of interest both due to its agricultural importance and to the current lack of knowledge concerning its underlying mechanism(s). The possibility of broad-spectrum resistance is particularly intriguing (Poland et al. 2009), again for both practical and theoretical reasons. One line of evidence for the existence of MDR or broad-spectrum QTL is based on co-localization of QTL identified in a range of different mapping populations (Jo et al. 2008; Wisser et al. 2006; Wisser et al. 2005; Yun et al. 2005). While these studies suggest that QTL for multiple diseases co-localize, inferences from previous QTL reports have been limited by the poor allelic sampling and low precision of QTL positions in most QTL studies.

In particular, the low resolution of most studies does not allow linkage to be distinguished from pleiotropy. MDR phenotype of a QTL can be attributed to the linkage of R-genes against different pathogens. In tomato, for example, a region on the short arm of chromosome 6 carries the *Cf-2* and *Cf-5* genes for leaf mold resistance (Dixon et al. 1998), the *Mi* gene for root knot nematode resistance (Milligan et al. 1998), and a number of major gene loci for resistance to other diseases (Dickinson et al. 1993; Zhang et al. 2002). Broad-spectrum resistance has also been associated with pleiotropic genes involved in mechanisms other than R-genes. Several genes involved in non-host resistance, basal resistance, systemic acquired resistance, and defense signaling pathways, are known to condition MDR [eg. *mlo* in barley (Buschges et al. 1997); *RPW8.1* and *RPW8.2* in *Arabidopsis* (Wang et al. 2007b); *npr1* in *Arabidopsis* (Cao et al. 1998); *Lr34* in wheat (Krattinger et al. 2009)]. Whether MDR is conditioned by typical R-genes or non-R-genes has implications for the durability and performance.

To capture and characterize diverse alleles associated with MDR in maize, heterogeneous inbred family (HIF) analysis was explored as an alternative to conventional QTL mapping and near isogenic line (NIL) development. Classical studies of disease QTL mapping involve developing mapping population(s) from crosses between resistant and susceptible lines, evaluating DNA marker genotypes and phenotypes of interest, and analyzing marker-trait association (Young 1996). Recombinant inbred lines (RILs) are widely used in QTL mapping, and have been reported to provide the best statistical power for QTL detection (Kaeppeler 1997). However, the development and genome-wide genotyping of the RIL population are time-consuming, and the use of RILs for detailed phenotyping or genetic dissection is not feasible. QTL characterization has usually been conducted using NILs, in which a given chromosomal segment is transferred from the donor genotype to the recurrent

genotype through consecutive generations of backcrossing. HIF analysis was first demonstrated as a useful approach for developing the pair of NILs that are isogenic at the majority of loci, but differ at a specific QTL, using intermediate materials easily achieved from general breeding programs (Tuinstra et al. 1997). It has been applied to validate the position and effect of QTL (Balasubramanian et al. 2009; Borevitz and Chory 2004; Kobayashi et al. 2006; Loudet et al. 2005; Njiti et al. 1998; Pumphrey et al. 2007), but to the authors' knowledge, HIF analysis has not been used for the analysis of allelic series for targeted loci.

This study was undertaken to extract alleles at loci associated with broad-spectrum resistance from a range of maize genotypes. From the genetic materials available to us, we chose to work on the HIFs derived from the crosses of B73 x CML52 and S11 x DK888, which allowed the investigation of maximum five alleles per locus (DK888 is a hybrid line). The tropical lines CML52 and DK888 were selected as the MDR donors on the basis of their superior resistance to northern leaf blight (NLB), southern leaf blight (also known as southern corn leaf blight, hereafter referred to as SLB), gray leaf spot (GLS), and other diseases (Kraja et al. 2000) (P. Balint-Kurti, pers. comm.). The consensus map of resistance loci in maize (Wisser et al. 2006) was used as a reference for genomic regions associated with disease QTL clusters. The study was dedicated not only to test the hypothesis that chromosomal regions where disease QTL co-localized harbor gene(s) controlling MDR (eight diseases evaluated), but also to generate genetic stocks suitable for uncovering the genetic basis of disease resistance and MDR. We conducted a parallel study of conventional QTL mapping, evaluating a population of RILs from the cross of B73 x CML52 for resistance to four diseases. The RIL-based QTL mapping also allowed empirical assessment of the advantages and drawbacks of using HIFs in analyzing targeted QTL.

MATERIALS AND METHODS

1. Plant materials

Two maize genotypes were chosen as donors of multiple disease resistance for the development of genetic materials. The first was CML52, a tropical inbred line developed by the International Maize and Wheat Improvement Center (CIMMYT); the second was DK888, a single-cross hybrid developed by Thailand Charoen Seeds Group collaborated with US Dekalb Seeds (Ekasingh et al. 2001). For conventional QTL mapping, a population of recombinant inbred lines (RILs) derived from CML52 crossed to the B73 inbred was used. It is one population from the larger Nested Association Mapping (NAM) population provided by The Maize Diversity Project (Buckler et al. 2009; McMullen et al. 2009; Yu et al. 2008). The RIL population consisted of 196 F₆ lines, though not all lines were evaluated for all diseases, which were sib- or self-mated for two generations to increase seed for testing. All lines were genotyped with 1106 single nucleotide polymorphism (SNP) markers, of which 773 were polymorphic and used for QTL mapping. Further details on the RILs are publicly available at www.panzea.org and www.maizegenetics.net.

For HIF (heterogeneous inbred family) analysis (Tuinstra et al. 1997), intermediate materials from the development of RILs were used. The overall strategy for HIF-based targeted QTL analysis is outlined in Fig. 2.1. The starting materials included 19 F₅ families from the cross of B73 x CML52 and 17 F₆ families from the cross of S11 x DK888, provided by The Maize Diversity Project and The USDA Germplasm Enhancement of Maize (GEM) Project (Balint-Kurti et al. 2006; Goodman 2005; Lee and Hardin 1997) respectively. Based on the maize disease QTL consensus map (Wisser et al. 2006), 39 bins associated with previously reported QTL for resistance to NLB, SLB, GLS and other diseases were selected as the candidate MDR regions.

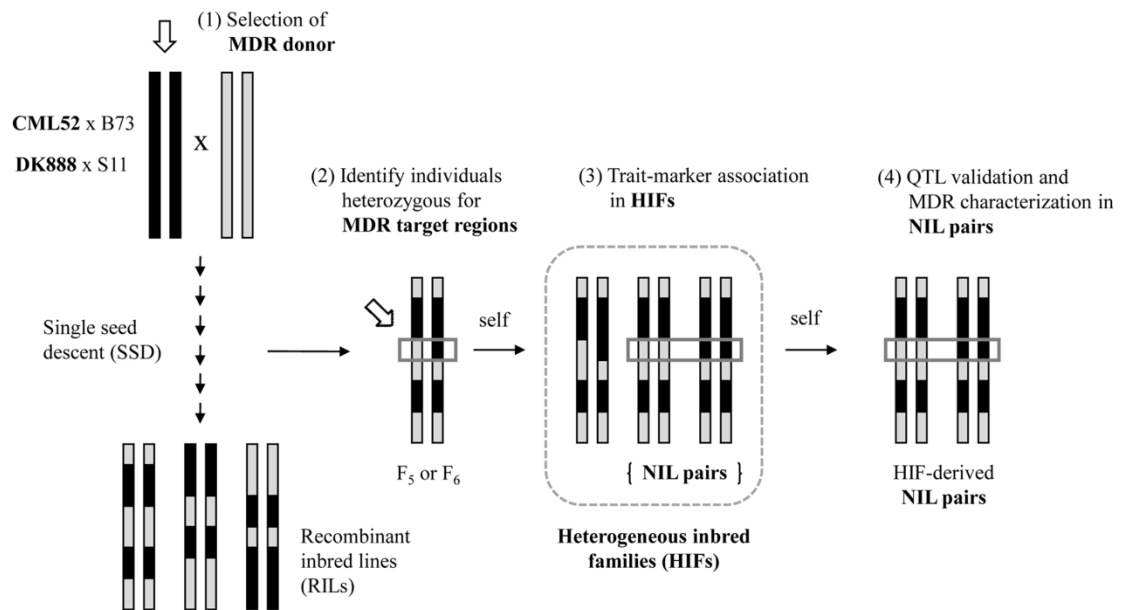


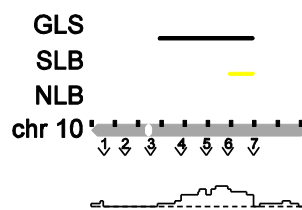
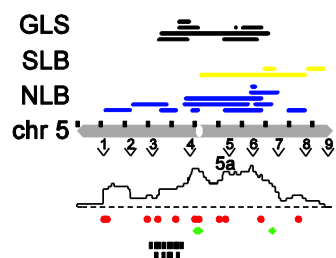
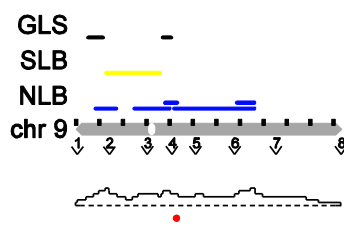
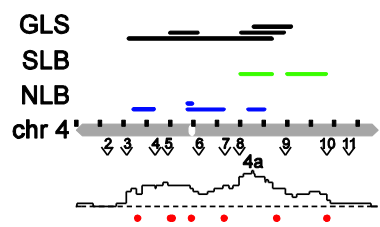
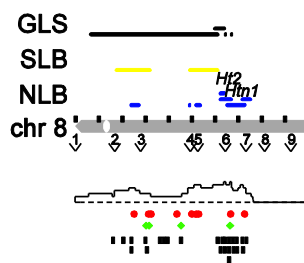
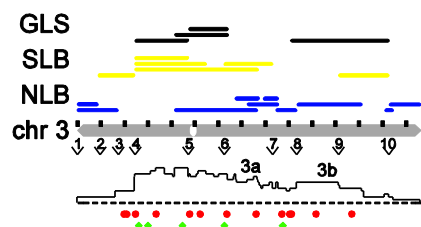
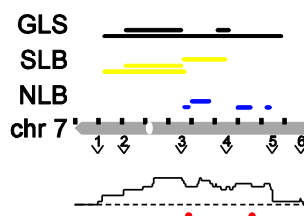
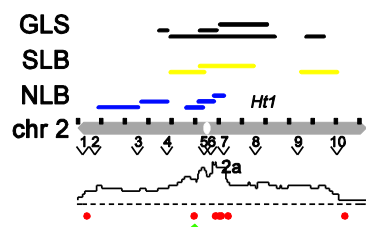
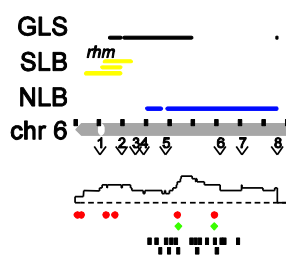
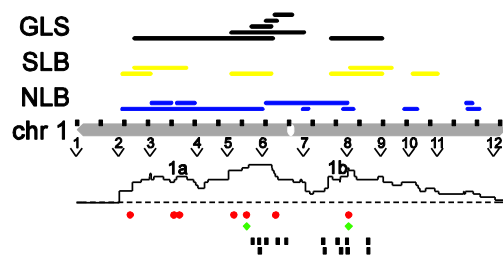
Figure 2.1 Strategy for identifying loci associated with multiple disease resistance (MDR) using heterogeneous inbred families (HIFs). (1) Various maize genotypes were screened for a number of important corn diseases. The genotypes CML52 and DK888 showed good level of resistance to multiple diseases, thus were chosen as MDR donors. (2) The nearly fixed F₅ and F₆ lines derived during the development of recombinant inbred line (RIL) populations were tested with markers covering the regions of MDR interest (the marker positions and the target regions are shown in Fig. 2). (3) In each derived F_{5:6} or F_{6:7} HIF, loci conditioning traits can be identified if pairs of near-isogenic lines (NIL pairs) showed significantly different phenotypes. (4) Sets of NIL pairs in more isogenic backgrounds can be derived by advancing selected lines of the HIF. The specific QTL isolated in NIL pairs can be subsequently tested for their MDR effects.

These regions of interest were targeted using 73 simple sequence repeat (SSR) markers (Fig. 2.2). For the B73 x CML52 materials, a full set of 94 individuals in 19 F₅ families (4-5 individuals per family) were initially tested for residual heterozygous loci, using 33 SSR markers covering 18 bins of interest. A subset of 43 F_{5:6} HIFs from 13 F₅ families were generated and tested for additional 31 SSR markers covering 20 more bins. The rest of the 51 F₅ lines were not advanced to F₆ because of poor agronomic performance. As a total, 64 SSR markers targeting 38 bins associated with MDR were applied on 43 F_{5:6} HIFs from the cross of B73 x CML52. For the S11 x DK888 materials, 46 F_{6:7} HIFs were developed by selfing 1-4 individuals from each of the 17 F₆ families. Residual heterozygous loci were detected in the 46 F_{6:7} HIFs with 17 SSR markers, focusing on 11 bins of interest. The subsequent derived NILs were generated by single-seed descent from 8 selected B73 x CML52 HIFs and 6 selected S11 x DK888 HIFs segregating for candidate NLB QTL or MDR hotspot regions. In this study, “NILs” refers to sets of HIF-derived F_{5:7}, F_{5:8}, F_{6:7}, F_{6:8} or F_{6:9} lines that contrasted for genetic regions of interest but were presumably isogenic at > 98 % of the genome (theoretical heterozygosity in the F₇ generation is 1.5625%).

2. Disease evaluations

Northern leaf blight. NLB trials were conducted in Aurora, New York in 2005-2008. Plants at the five to six-leaf stage were inoculated with *Setosphaeria turcica* (anamorph *Exserohilum turcicum*) race 1 (isolate EtNY001, isolated from infected leaf collected at Freeville NY in 1983). Spore suspension (0.5ml of 4 x 10³ conidia per ml in 0.02% Tween 20) and colonized sorghum grains (1/4 teaspoon, ~1.25 ml) were placed in the whorl of each plant. *S. turcica* was cultured on lactose – casein hydrolysate agar (LCA) for 2-3 weeks, under a 12 hr/12 hr normal light-dark cycle at room temperature. Liquid inoculum (spore suspension) was prepared by dislodging the

Figure 2.2 The markers targeting chromosomal regions associated with clusters of disease QTL (quantitative trait loci for disease resistance) in maize. The disease QTL consensus map was adapted from the study of Wisser et al. (2006). The 10 maize chromosomes and standard bin positions are shown, with arrowheads and the numbers indicating the start of each given bin. Previously reported QTL for resistance to northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS) are shown as blue, yellow, and black bars above the chromosomes. The histogram below each chromosome represents the frequency of QTL (per cM on the IBM2n map) for resistance to NLB, SLB, GLS, common rust, downy mildew, common smut, ear and stalk rot, *Aspergillus flavus* (aflatoxin), Stewart's wilt, and viral diseases. In the present study, 73 simple sequence repeats (SSR) markers (red circles and green diamonds at the very bottom of the chromosomes) were used to target 39 bins associated with disease QTL clusters. For the 94 lines in 19 F₅ families derived from B73 x CML52, 64 SSR markers (red circles) were used to target 38 bins. For the 46 lines in 17 F₆ families derived from S11 x DK888, 17 SSR markers (green diamonds) were used to target 11 bins. Additional 66 SSR markers (black rectangles at the very bottom of the chromosomes) were used to fine-localize the candidate QTL.



conidia from the plates with sterilized ddH₂O, filtering the suspension through 4 layers of cheesecloth, then adjusting the concentration with the aid of a haemocytometer. Solid inoculum was prepared by inoculating autoclaved sorghum grains with the suspension of spores and mycelium dislodged from heavily colonized LCA plates. To prevent caking, the inoculated sorghum grains were shaken every day until use. A number of disease components, including incubation period (IP), lesion number (LN), primary diseased leaf area (PrimDLA), diseased leaf area (DLA) and area under the disease progress curve (AUDPC), were used to evaluate the resistance to NLB. IP was rated for individual plants as the number of days after inoculation when a plant started showing wilted lesions. LN was rated for individual plants at 2-3 weeks after inoculation, as the total number of lesions on a plant. PrimDLA was rated on a row basis at 2-3 weeks after inoculation, as the percentage of infected leaf area of the inoculated leaves. DLA was rated as the percentage of infected leaf area of the entire plant, disregarding decayed bottom leaves. In each season, 3-4 DLA scores were taken at 10 to 14 day-intervals, starting from 1-2 weeks after the onset of secondary infection. The DLA scores were used to calculate $AUDPC_{DLA} = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})(t_{i+1} - t_i)}{2}$, where y_i = DLA at time i , $t_{i+1} - t_i$ = day interval between two ratings, n = number of ratings (Gaurilcikiene et al. 2006).

Southern leaf blight. SLB trials were conducted in Clayton, North Carolina in 2007 and 2008, and Homestead, Florida in 2007. Plants at the four to six-leaf stage were inoculated with *Cochliobolus heterostrophus* race O (isolate 2-16Bm) as previously described (Carson 1998; Carson et al. 2004). Disease severity was rated based on a 1 to 9 scale corresponding to the diseased leaf area on primarily the ear leaf. Four to six times of evaluation were taken at 5 to 10-day intervals from around 2 weeks after anthesis. The disease severity scores were used to calculate $AUDPC_{Severity} = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})(t_{i+1} - t_i)}{2}$, where y_i = disease severity at time i , $t_{i+1} - t_i$ = day interval between

two ratings, n = number of ratings.

Gray leaf spot. GLS trials were conducted in Andrews, North Carolina in 2007-2008, and Blacksburg, Virginia in 2008. Experiments were performed at the non-tillage fields located in the valleys with regular morning mists and heavy dews which favor the development of GLS (caused by *Cercospora zea-maydis* and/or *Cercospora zeina*). For the HIFs and NILs, disease severity was rated as described for SLB, with a minimum of three ratings per season. For the population of RILs, disease severity was scored based on a 1 to 5 scale with 0.25 increments, according to the disease progress on the ear leaf (Saghai Maroff et al. 1993). The evaluation was conducted four times at 7 to 8-day intervals starting around 2 weeks after anthesis. The AUDPC_{Severity} was calculated as described above.

Anthraxnose stalk rot. ASR trials were conducted in Aurora, New York in 2007-2008. Plants were inoculated with *Colletotrichum graminicola* (teleomorph: *Glomerella graminicola*) (isolate Cg151, obtained from Gary Bergstrom of Cornell University) at anthesis stage [$> 50\%$ of the plants in every row were tasseling (Keller and Bergstrom 1988)]. Inoculum was cultured on oatmeal agar for 2 weeks under fluorescent light at room temperature (Muimba-Kankolongo and Bergstrom 1990), and prepared as described for NLB. Each plant was punctured at the first internode above the brace root with an ice pick, inserted with a 1 ml pipette tip, and inoculated with 1 ml of 10^6 conidia per ml (0.02% Tween 20) through the tip. Resistance to ASR was evaluated by splitting the stalks longitudinally at 4 weeks after inoculation. Total diseased internode area (ASR %) was rated for individual plants, as the sum of the percentages of discolored area of individual internodes (Keller and Bergstrom 1988). For the HIFs and NILs, in 2007, six plants per row were inoculated and eight consecutive internodes per plant were scored; in 2008, eight plants per row were inoculated and six consecutive internodes per plant were scored. For the population of

RILs, ASR inoculation was conducted in NLB plots in 2007 and 2008. For each row, four plants were inoculated and six consecutive internodes per plant were scored. Data from all the scored internodes were summed for analysis, except for the B73 x CML52 RILs in 2008, only internodes 1 to 5 were used to calculate ASR %.

Anthraxnose leaf blight. ALB trials were conducted in Aurora, New York in 2007. Plants were inoculated at the five to six-leaf stage with *Colletotrichum graminicola* (teleomorph: *Glomerella graminicola*). Inoculum was cultured on oatmeal agar for 2 weeks under fluorescent light at room temperature (Muimba-Kankolongo and Bergstrom 1990). Spore suspension (0.5ml of 2×10^4 conidia per ml in 0.02% Tween 20) and colonized sorghum grains (1/4 teaspoon, ~1.25 ml) were placed in the whorl of each plant. The preparation of liquid and solid inoculum, and the ratings of IP, DLA and AUDPC_{DLA} was as described for NLB.

Common rust. Resistance to rust was evaluated in Aurora, New York in 2007-2008. In 2007, plants in NLB plots were evaluated for rust symptoms caused by natural infection. In 2008, plants at the six to eight-leaf stage were inoculated with isolate(s) of *Puccinia sorghi*, which were collected from naturally infected leaves harvested at the same location in 2007. To increase inoculum, three to four-leaf stage seedlings of susceptible sweet corn were sprayed with urediniospore suspension (~300 mg stock spores in 100 ml Sortrol oil) (Webb et al. 2002), incubated at a mist chamber (> 85% RH) overnight, and kept in the greenhouse for 2-3 weeks until use. Inoculum was prepared by agitating infected leaves with matured rust pustules in distilled water and filtering through four layers of cheesecloth. Each field plant was inoculated at two time points 10 days apart with 1 ml spore suspension (2×10^5 urediniospores per ml in 0.02% Tween 20) in the whorl (Pataky and Campana 2007). Disease severity was rated based on a 0-10 scale with 0.5 increments, corresponding to the percentage of infected leaf area of the entire plant. The severity scores were taken twice and

averaged in 2007, and were evaluated three times at 9-day intervals from four weeks after inoculation in 2008. The AUDPC_{Severity} was calculated for the 2008 data as described above.

Common smut. Resistance to smut was evaluated in Aurora, New York in 2007-2008. In 2007, plants in NLB plots were evaluated for naturally occurring smut galls. In 2008, plants were inoculated with six compatible strains of *Ustilago maydis* (UmNY001, UmNY002, UmNY003, UmNY004, UmNY008 and UmNY009), which were isolated from naturally infected smut galls collected at the same location in 2007 [isolation procedure: (Thakur et al. 1989); compatibility test: (Puhalla 1968)]. Sporidia of compatible strains were cultured separately in 10 ml of potato-dextrose broth (PDB) on a shaker at 100 rpm at room temperature for 1 day, transferred to flasks of 50 ml PDB, then incubated at 100 rpm overnight. The sporidial suspension was diluted to a final concentration of 10^6 sporidia per ml (0.02% Tween 20) with sterilized ddH₂O. Inoculation was conducted when the silks of most ears in a row had emerged 1-5 cm. Equal amounts of sporidia from compatible isolates were mixed right before inoculation, and 2 ml of sporidial suspension (10^6 sporidia per ml in 0.02% Tween 20) was injected into the shoot-bagged first ears of the plants through silk channel (du Toit and Pataky 1999). The incidence and severity of the development of ear galls and stalk galls were rated at 4-5 weeks after anthesis. Severity scores were evaluated for individual plants on a 0-10 scale, corresponding to the number and size of galls, and the disease severity of the entire plant.

Stewart's wilt. Plants at the five to six-leaf stage were inoculated with *Pantoea stewartii* (Syn. *Erwinia stewartii*) strain PsNY003 (obtained from Helene Dillard of Cornell University) following a modified pinprick method (Blanco et al. 1977; Chang et al. 1977). Bacteria was first cultured on nutrient agar plates at room temperature under a 12 hr/12 hr normal light-dark cycle for 2 days, then transferred to nutrient agar

broth and shaken overnight at 100 rpm. The inoculum was quantified using a haemocytometer, and adjusted to a final concentration of 10^7 colony forming units per ml with sterilized 0.1 M NaCl solution (Suparyono and Pataky 1989). Plants were inoculated by piercing the whorl leaves twice with a multi-pin inoculator pre-dipped in bacterial suspension. The inoculator was a tong with 30 T-pins (1.5 inch long), pieces of sponge (5.5 cm x 6.5 cm) and cork board (3/8 inch thick) fastened on its two arms. Primary diseased leaf area was rated as the percentage of infected area of the inoculated leaves. It was evaluated twice at 7-day interval from two weeks after inoculation.

3. DNA extraction and genotyping

A modified min-prep CTAB method (Doyle and Doyle 1987; Qiu et al. 2006) was used for genomic DNA extraction. About 0.1 g frozen leaf tissue was ground with a stainless steel ball (5/32 inch diameter, OPS Diagnostics, NJ, USA) in each well of a 96-well plate (Corning® Costar 96 Well Polypropylene Cluster Tubes), at 450 strokes/min for 50-120 sec using Genogrinder 2000 (SPEX CertiPrep Inc.). Pulverized sample was suspended in 500 µl of CTAB extraction buffer [2 % (w/v) hexadecyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.2% (v/v) of 2-mercaptoethanol; 2-mercaptoethanol was added prior to use]. After incubating at 65 °C for 30-50 min, 400 µl of chlorophorm/isoamyl alcohol (24:1, v/v) was added and mixed thoroughly with CTAB buffer. Samples were centrifuged at 5200 rpm for 15 min, and the supernatant was transferred to a new tube. DNA was precipitated by incubating the mixture of the supernatant and 300 µl of isopropanol at -20 °C overnight, and centrifuged at 5200 rpm at 4 °C for 12 min. DNA pellet was recovered by desalting with 70% then 100% ethanol, air-drying, and dissolving in 100-150 µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Simple sequence repeat (SSR) markers were used for genotypic analysis. A modified single-reaction nested PCR method using the specific primer pair along with a fluorescently-labeled universal primer (Schuelke 2000), was applied to incorporate fluorescent dye in PCR product. Each PCR reaction was performed in a total volume of 13 μ l, containing final concentrations of 1x PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% (v/v) Triton X-100], 1.5 mM MgCl₂, 1 M betaine, 0.16 μ M fluorescently-labeled universal M13(-21) primer, 0.04 μ M forward-specific primer with M13(-21) tail at its 5' end, 0.16 μ M reverse-specific primer, 1-3 units Taq polymerase, and 20-50 ng template DNA. The thermal cycling parameters were: 1 cycle of 94°C (5 min), 30 cycles of 94 °C (30 sec) / 56 °C (45 sec) / 72 °C (45 sec), 8 cycles of 94 °C (30 sec) / 53 °C (45 sec) / 72 °C (45 sec), and the final extension at 72 °C (10 min). The resulting DNA fragments labeled with different fluorescent dyes were multiplexed (0.7 μ l PCR product per specific primer pair, up to four PCR reactions were combined), mixed with 0.05-0.1 μ l GeneScan-500 LIZ size standard and 9 μ l formamide, and analyzed on the Applied BioSystems 3730xl DNA Analyzer at Biotechnology Resource Center at Cornell University. The sizes of amplicons were scored in GeneMapper v. 3.0.

4. Experimental design

HIFs and NILs. To identify QTL in HIFs, lines of derived HIFs were individually genotyped for segregating marker loci, and phenotyped for different disease traits. About 50-140 individuals per family were analyzed, according to the availability of seeds. Individual plants in a HIF were grown within a single block with two B73 rows as the borders. For QTL confirmation, NILs of each set (NILs derived from a parental line) were randomized within a block, with two B73 rows as the borders. To investigate resistance levels of genetic backgrounds in different HIFs, 34

F_{5:6} HIFs derived from 14 F₅ of B73 x CML52 were evaluated in 2006 for NLB (Aurora, NY), SLB (Clayton, NC) and GLS (Andrews, NC), and 16 F_{6:7} HIFs derived from 6 F₆ of S11 x DK888 were evaluated in 2007 for SLB (Clayton, NC) and GLS (Andrews, NC). Each HIF was put in rows in each of the two replications. For the NLB trial, the resolvable incomplete block design (an alpha design; Patterson and Williams 1976) was used, with 2 replications, 6 blocks per replication, and 6 HIF rows plus 2 B73 control rows per block. In the SLB and GLS trials, HIFs derived from the same parental line were grown next to each other, for better visual comparison in the field.

RILs. The RILs were evaluated for NLB and ASR in Aurora, NY in 2007 and 2008. Lines were planted in a 7 foot row and thinned to 8-10 plants per row. The trial was conducted using a single replication with an augmented design, using each of the inbred parents (B73 and CML52) as repeated checks. Incomplete blocks consisted of 18 RILs and each of the two parents.

For NLB, three disease severity ratings were taken each season and a multivariate mixed model was used to obtain Best Linear Unbiased Predictions (BLUPs) of NLB disease severity at each time point. The trait distribution showed a very skewed distribution, so a square root transformation was used on the raw data for NLB prior to further analysis. The model is as follows:

$$D_t = Y_i + B_{j(i)} + L_k$$

Where D is the disease severity at time t , Y_i is the random effect of year i , B_j is the random effect of block j in year i , and L_k is the random effect of line k . Model solution provided BLUPs for NLB disease severity at each of the three time points. The BLUPs were used to calculate AUDPC as described above and used for QTL analysis.

For ASR, 4 plants were inoculated and individually evaluated for disease severity at internodes 1-6. A similar multivariate mixed model was used for obtaining the

BLUPs for ASR:

$$D_n = Y_i + B_{j(i)} + L_k$$

Where D is the disease severity for internode n , Y_i is the random effect of year i , B_j is the random effect of block j in year i , and L_k is the fixed effect of line k . The model solution provided Best Linear Unbiased Estimates (BLUEs) for ASR severity at each internode. The disease severity values for internodes 1-5 were summed and subsequently used for QTL mapping.

For GLS evaluation, lines were evaluated in Blacksburg, VA in 2008. Lines were planted in 12 foot long single row plots, with 12-14 plants per row. The trial was arranged in an augmented design with a single replication. Incomplete blocks consisted of 18 RILs and each of the two parents. Lines were evaluated for disease severity at four time points. The model for field effects is:

$$D_t = B_j + F_k + L_k$$

Where D is the disease severity at time t , B_j is the random effect of block j , F_k is the random effect of flowering time for line k , and L_k is the fixed effect of line k . Flowering time (days to anthesis) for each line was included in this model, as it was found to have a highly significant correlation with disease severity for GLS. AUDPC for GLS severity was calculated as described above.

Resistance to SLB was evaluated in 3 environments. In each trial the same augmented design with a single replication was used. Disease severity was scored on a row basis at two time points. A similar multivariate mixed model was used for obtaining the BLUPs for SLB:

$$D_t = E_i + B_{j(i)} + L_k$$

Where D is the disease severity at time t , E_i is the random effect of environment i , B_j is the random effect of block j in environment i , and L_k is the fixed effect of line k . The model solution provided BLUPs for SLB severity at the two time points. These

values were averaged and subsequently used for QTL mapping.

5. Data analysis

QTL identification in HIFs. Analysis of variance (ANOVA) was conducted on an individual trait-marker basis in each of the segregating HIFs using JMP 7.0. The markers associated with disease traits were identified when significant phenotypic differences were detected between homozygous genotypes carrying contrasting parental alleles, according to two-tailed Student's *t* test at $P < 0.05$. For the HIFs segregating for multiple markers, the Bonferroni correction method was used to adjust the significance threshold levels. The mixed stepwise regression, with a significance probability of $P < 0.05$ for each marker to enter/leave the model (van Dam et al. 2003), was also performed in JMP 7.0 to confirm the effects of identified markers.

QTL identification in NILs. Each set of NILs was analyzed separately using JMP 7.0. Phenotypes of a set of NILs were first tested by fitting a linear least squares model with “genotype” and “replication” as fixed factors. In the cases of NILs being evaluated in both 2007 and 2008, the variables “genotype”, “year” and “replication (year)” were used in the model. If no phenotypic difference was found among the NILs (genotype effect is nonsignificant), none of the target markers in the NIL set were considered QTL, and no further analysis was conducted. When significant difference in least squares means of the NILs was detected within the set ($P < 0.05$), markers at which the NILs differed were analyzed for their associations with disease traits, on an individual trait-marker basis. A linear least squares model with “marker” and “replication” as fixed factors was performed. Phenotypic difference between least squares means of contrasting homozygous genotypes at the target marker was determined by two-tailed Student's *t* test at $P < 0.05$. Markers significantly associated with traits were candidate disease QTL. The marker with greatest significance in a

linked block was considered to reflect the most likely QTL position. For the sets of NILs segregating for more than one candidate QTL, potential correlation and/or interaction between the unlinked significant markers were being analyzed, following a series of statistical tests described by Szalma *et al.* (2007). Briefly, the NILs were grouped by the alleles of each of the significant marker loci. Trait-marker analysis was conducted for the NILs fixed for one marker but contrasting for the other marker(s). A QTL was declared if the lines contrasting for this locus were significantly different from each other ($P < 0.05$), given the other potential QTL in the genome being fixed.

QTL identification in RILs. Three types of interval mapping were employed for QTL mapping. Composite interval mapping (CIM), inclusive composite interval mapping (ICIM) and Bayesian interval mapping (BIM) were conducted using QGene 4.2.3 (Joehanes and Nelson 2008). Due to the similarity between CIM and ICIM, only the ICIM results are presented here. For ICIM, the default scan interval of 2 cM was used. A stepwise cofactor selection was used with a maximum of 8 markers selected and a selection threshold of $F = 2$. For BIM, a 2.5 cM scan interval was used. For phenotypic correlations, Pearson correlation coefficients were found using PROC CORR in SAS 9.1. For each of the traits the BLUP values from each trait were used to determine correlations between resistance to the different diseases.

RESULTS

1. Selection of MDR genotypes

As a first step towards testing for MDR loci, genotypes with resistance to multiple diseases had to be identified (Fig. 2.1). The selection of MDR donors was based on a review of documented resistance performance of various maize genotypes and confirmation through field evaluation. Among the parental lines of the HIFs to which we had access, CML52 and DK888 were the best MDR genotypes. As shown

in Table 2.1, CML52 conferred high levels of resistance to NLB, SLB, and GLS among 253 diverse lines in the maize association population (Liu et al. 2003). In terms of MDR, CML52 was in the top 5% of lines analyzed (R. Wisser, J. Kolkman, R. Nelson and P. Balint-Kurti, pers. comm.), and had a high level of resistance against ear rot among the 394 tropical lines developed by the International Maize and Wheat Improvement Center (CIMMYT; http://www.cimmyt.org/english/wps/obtain_seed/germplas.htm). CML52 also showed strong resistance against the colonization and sporulation of *Aspergillus flavus*, as well as the accumulation of aflatoxin in a smaller set of diverse maize lines (S. Mideros, pers. comm.). DK888, on the other hand, was overall the best genotype carrying favorable alleles for resistance to NLB, SLB, GLS, northern leaf spot (NLS, caused by *Cochliobolus carbonum*, anamorph *Bipolaris zeicola*), and common rust in a study conducted as part of the Genetic Enhancement of Maize program of the USDA (Kraja et al. 2000).

Plant maturity has been found to be highly correlated with disease resistance (Bubeck et al. 1993; Carson 1999; Keller and Bergstrom 1988; Leonard and Thompson 1976). Late-flowering maize lines tend to be more resistant to NLB, SLB and GLS. In the maize association panel, 48%, 45% and 52% of resistance variation to NLB, SLB and GLS, respectively, were explained by variation in days to anthesis (R. Wisser, J. Kolkman, R. Nelson and P. Balint-Kurti, pers. comm.). Given the recognized relationship between disease resistance and maturity, it is important to acknowledge that both CML52 and DK888 were late-maturing. On average, the number of days to anthesis (in Aurora NY) for CML52 and DK888 are both ~100 days after planting, which is around 21 days later than the median flowering time of the maize association population (J. Kolkman, pers. comm.), and ~26 days later than the

Table 2.1 Previous evidence regarding the resistance properties of CML52 and DK888. CML52 and DK888 were selected as potential donors of multiple disease resistance (MDR) alleles based on their superior resistance to northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS), ear rot, northern leaf spot (NLS), common rust, and/or *Aspergillus flavus*.

Diseases	CML52		DK888	
	Resistance ranking	Reference	Resistance ranking	Reference
NLB	14 th out of 253 diverse lines ^a	Wisser et al., pers. comm.	1 st out of 34 lines ^c	Kraja et al. 2000
SLB	38 th out of 253 diverse lines ^a	Wisser et al., pers. comm.	1 st out of 34 lines ^c	Kraja et al. 2000
GLS	10 th out of 253 diverse lines ^a	Wisser et al., pers. comm.	2 nd out of 34 lines ^c	Kraja et al. 2000
Ear rot	5 th out of 394 CIMMYT tropical lines ^b	CIMMYT ^b	na	na
NLS	na	na	4 th out of 34 lines ^c	Kraja et al. 2000
Common rust	na	na	3 rd out of 34 lines ^c	Kraja et al. 2000
Aspergillus Ear rot	3 rd out of 18 diverse lines with respect to the resistance against the colonization of <i>A. flavus</i> on maize kernels	Mideros et al. 2008	na	na
	1 st out of 18 diverse lines with respect to the resistance against aflatoxin accumulation			
	2 nd out of 7 potentially resistant lines with respect to the resistance against the sporulation of <i>A. flavus</i> on developing maize kernels	S. Mideros, pers. comm.		

^a The maize association population consisting of panel of 253 diverse lines (Liu et al. 2003) were evaluated in multi-environments for multiple years (R. Wisser, J. Kolkman, and P. Balint-Kurti, pers. comm.). The ranking was based on the genotypic breeding values from a multivariate mixed model, accounting for maturity and population structure. CML52 was overall the 11th best MDR line for resistance to NLB, SLB and GLS.

^b Resistance to ear rot (causal agent was not specified) was evaluated on 394 out of 539 CIMMYT Maize Lines (CMLs) developed by the International Maize and Wheat Improvement Center (CIMMYT). While disease scores for the 394 CML lines ranged from 1-5, CML52 was rated as 1.5. (URL: http://www.cimmyt.org/english/wps/obtain_seed/germplas.htm)

^c The ranking was based on the relative frequencies of favorable alleles in the populations derived from 34 different germplasm sources, including 7 tropical hybrids, 12 temperate accessions and 15 tropical populations. The allele frequencies were estimated based on a two-year field trial using Dudley's theory. DK888 was overall the best genotype carrying favorable alleles for resistance to NLB, SLB, GLS, NLS and common rust.

elite inbred line B73. To examine if the resistance is the result of developmental effects rather than defense mechanisms, juvenile plants of CML52 and DK888 were evaluated in repeated greenhouse and field trials for NLB, the primary emphasis of disease at the inception of this study. Compared to B73, the formation of NLB lesions in juvenile plants of CML52 and DK888 was delayed by around 4 and 5 days in the greenhouse, and around 4 and 9 days in the field, respectively (data not shown). The results suggested that NLB resistance in both lines is effective regardless of their maturity stage or growing environment (greenhouse versus field condition). The associations between maturity and resistance effects for individual chromosomal segments are reported below.

The above lines of evidence led to the choice of CML52 and DK888 as desirable sources of MDR alleles. Data subsequently collected from B73, CML52, DK888 and derived HIFs and NILs further supported the superior resistance of CML52 and DK888 to various diseases (Table 2.2). (The maximum, median and minimum trait values of derived HIFs and NILs were used as a general reference for resistance and susceptibility. The data should be viewed with caution, as they are not representative of the overall disease phenotypes in maize.) DK888 consistently showed outstanding performance for resistance to all the diseases tested. CML52, however, showed good levels of resistance to NLB, SLB, GLS, ALB, rust and smut, but was moderately resistant to Stewart's wilt, and was moderately susceptible to ASR. For ALB and rust, to which CML52 and DK888 were both resistant, DK888 was significantly more resistant than CML52, suggesting that resistance alleles are more enriched and/or more effective in DK888. It is worth noting that B73 was moderately susceptible to most diseases, including NLB, SLB, GLS, ASR, ALB and Stewart's wilt, but was resistant to common rust and common smut, both caused by obligate biotrophic fungi. For smut, there were no naturally occurring stalk galls observed on B73. Even with

Table 2.2 Characteristics of the donor lines with respect to disease resistance and maturity. Resistance spectra of DK888, CML52, and B73, the lines used as donors of alleles in the study, were evaluated by testing their responses to a range of pathogens with diverse lifestyles. The diseases evaluated included northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS), anthracnose stalk rot (ASR), anthracnose leaf blight (ALB), common rust, common smut, and Stewart's wilt. DK888 conferred superior resistance to all the diseases. CML52 conferred high levels of resistance to NLB, SLB, GLS, ALB and common rust, all caused by fungal leaf pathogens. B73 was moderately susceptible to most of the diseases, but conferred high levels of resistance to common rust and common smut, both caused by biotrophic fungal pathogens.

Trait	Parameter	Maize genotype ^a			Derived HIFs and NILs ^b			Lifestyle of the causal agent			
		DK888	CML52	B73	Min	Med	Max	Parasitism	Colonized tissue	Vasculature colonization ^d	Reference
Maturity	Days to anthesis (days)	100 A	100 A	74 B	72	88	100				
NLB	AUDPC _{DLA} (%-day)	132 B	143 B	442 A	19	575	1225	Hemibiotrophic	Leaf	Y *	Jennings and Ullstrup 1957
SLB	AUDPC _{Severity} (severity-day)	8 B	20 B	67 A	8.5	15	57	Necrotrophic	Leaf	N	Jennings and Ullstrup 1957
GLS	AUDPC _{Severity} (severity-day)	46 B	49 B	92 A	44	67	115	Hemibiotrophic	Leaf	N	Beckman and Payne 1982
ASR ^c	Discolored internode area (Total % of internode)	62 B	162 A	160 A	9	140	580	Hemibiotrophic	Stalk ^c	Y ^c	Venard and Vaillancourt 2007a, b
ALB ^c	AUDPC _{DLA} (%-day)	133 C	228 B	400 A	121	432	752	Hemibiotrophic	Leaf ^c	Y ^c	Mims and Vaillancourt 2002; Venard and Vaillancourt 2007b
Common rust	AUDPC _{Severity} (severity-day)	20 B	34 A	31 A	38	74	99	Biotrophic	Leaf	N	Van Dyke and Hooker 1969
Common smut	Incidence of stalk gall (%)	0 A	0 A	0 A	0	0	0.38	Biotrophic	Leaf, stalk, ear, tassel	Y	Brefort et al. 2009
	Severity of stalk gall (scale)	0 A	0 A	0 A	0	0	3.3				
	Incidence of ear gall (%)	0.32 B	0.66 A	0.08 C	0	0.53	1				
	Severity of ear gall (scale)	0.6 A	0.8 A	0.1 B	0	2.1	6.6				
Stewart's wilt	Primary DLA (%)	10 C	31 B	44 A	5	55	75	Biotrophic	Systemic	Y *	Braun 1982; Koutsoudis et al. 2006

^a Trait values are least squares means, calculated from the linear least squares model with “maize genotypes”, “environment” and “replication nested within environment” as independent variables. Different letters represent significant differences among DK888, CML52 and B73 lines, determined by Tukey-Kramer HSD (honestly significant difference) test at $P < 0.05$.

^b The observed minimum (Min), median (Med) and maximum (Max) trait values among all the lines derived from the heterogeneous inbred families (HIFs) of B73 x CML52 and S11 x DK888 are shown. Trait values are least squares means, calculated from the linear least squares model with “maize genotypes”, “environment” and “replication nested within environment” as independent variables.

^c ASR and ALB are both caused by *Colletotrichum graminicola*. The pathogen can infect the leaf, stalk and root tissues of maize. *C. graminicola* also colonizes the vasculature, but mainly proceeds through the fiber cells.

^d The ability (Y) and inability (N) of the causal pathogen to colonize the vascular tissues of maize.

* The pathogen grows and progresses in the xylem lumen.

U. maydis sporidia artificially inoculated through the silk channel, the incidence and severity of ear galls was as low as 8% (0.1 on a scale of 0-10, with 3.3 as the highest observed in this experiment).

2. Identifying individuals heterozygous for MDR target regions

To isolate disease QTL regions in NILs, 64 SSR markers were selected to target 38 bins (of the 100 bins of the maize genome, ~20 cM per bin) associated with identified QTL for resistance to primary NLB, GLS, SLB, and to other diseases [Fig. 2.2; adapted from the study of Wisser et al. (2006)]. In the disease QTL consensus map in maize, NLB QTL were identified in 41 bins on chromosomes 1 to 9, and GLS QTL and SLB QTL were found in 30 of these 41 bins. Among the 38 target bins, NLB QTL, GLS QTL, and SLB QTL were reported in 25, 25, and 17 bins, respectively. To enhance the likelihood of capturing MDR QTL, markers were chosen to focus on the regions with greatest density of disease QTL. Higher marker densities were used for genomic regions carrying large numbers of disease QTL relative to gene density and the numbers of flowering time QTL (Wisser et al. 2006). Overall, the majority of the target bins corresponded to QTL for more than one of the three diseases (NLB, SLB and GLS).

As expected, the initial sample sets consisting of 94 F₅ derived from B73 x CML52 and 46 F₆ derived from S11 x DK888 allowed successful identification of heterozygous lines for almost every locus of interest. The determination of sample sizes was based on the expected heterozygosity per locus in the F₅ (6.25%) and F₆ (3.125%) generation. The heterozygosity level was used to estimate the percentage of heterozygous loci across the genome in an individual line, as well as the percentage of lines that are heterozygous for a single marker locus. As a result, in the full set of 94 individuals in 19 F₅ families derived from B73 x CML52, an average of 6.16% of the

marker loci were found to be heterozygous per line, and an average of 6.17 % of the lines were found to be heterozygous per locus. In the subset of 43 individuals in 13 F₅ families, the average percentages of “heterozygous loci per line” and “heterozygous lines per locus” are 7.27% and 7.44% respectively. In the set of 46 individuals in 17 F₆ families derived from S11 x DK888, an average of 6.14% of the markers were heterozygous in an individual line, and an average of 4.60% of the lines were heterozygous for each marker locus. All the ratios approximately conformed to the expected heterogeneity in the F₅ and F₆ generation.

3. NLB QTL identified in the HIFs derived from B73 x CML52 and S11 x DK888

The putative NLB QTL identified in the HIFs are listed in Table 2.3. From 2005 to 2006, 15 of F₆, 7 of F₇, and 2 of F₈ segregating families (a total of 24 HIFs) derived from B73 x CML52 were evaluated for resistance to NLB using different phenotypic parameters, including IP, LN, PrimDLA, DLA, and AUDPC. Out of 27 bins investigated, significant phenotypic contrasts between CML52 homozygotes and B73 homozygotes of the target loci were detected in bins 1.06, 1.07-1.08, 5.03, 6.05, and 8.02-8.03 (significance found at least within 2 families). By evaluating sets of NILs developed from selected lines in the HIFs, the resistance effects of CML52 alleles at bins 1.06 and 6.05, and B73 allele at bin 5.03 were validated. These QTL coincided with NLB QTL previously detected in two other maize populations: B52 x Mo17 [5.02-5.03: Mo17 as resistance donor (Freymark et al. 1993)], D32 x D145 [1.06-1.08: D32, 5.03-5.04: D145, 6.05-6.08: D145 as resistance donors (Welz et al. 1999b)]. Bins 1.07-1.08 and 8.02-8.03 for NLB resistance were respectively detected in 2 HIFs of B73 x CML52. However, these two regions were not declared as NLB QTL, as their effectiveness was not verified in derived NILs.

In 2006, three F₇ HIFs derived from S11 x DK888 were evaluated for NLB resistance. Out of five bins tested, the DK888 allele(s) at bin 8.05-8.06 was found effective for delaying lesion formation by ~6 days in two HIFs. The resistance effect was further confirmed in a set of 8 NILs for IP, DLA and AUDPC (Table 2.3). Bin 8.05-8.06 has been described as a region associated with quantitative and qualitative resistance to NLB (Welz and Geiger 2000). The NLB QTL at bin 8.05-8.06 were previously identified in the mapping populations of Lo951 x CML202 [CML202 as resistance donor (Schechert et al. 1999; Welz et al. 1999a)] and D32 x D145 [D32 as resistance allele (Welz et al. 1999b)]. Two race-specific major genes for NLB resistance, *Ht2* and *Htn1*, were also mapped to this complex region in the populations of A619Ht2 x W64A (Zaitlin et al. 1992), W22*Htn1* x A619Ht2 and W22*Htn1* x A619Ht1 (Simcox and Bennetzen 1993).

To better localize the candidate NLB QTL identified in HIFs, 66 flanking SSR markers (six for bin 1.06, eight for bin 1.07-1.08, 14 for bin 5.03, 18 for bin 6.05, 5 for bin 8.02-8.03, and 15 for bin 8.06) were used to estimate the start and end points of heterozygous loci in F₅ and F₆ families of B73 x CML52 and S11 x DK888, respectively. The map interval of each QTL is assumed to extend halfway between two markers around each end of the QTL. The identified QTL have an average size of 85 cM on the IBM2n map [~21 cM on an F₂ map (Lee et al. 2002)], and ~28 Mb on the physical map. The accuracy of the estimation was affected by the numbers of flanking markers used to determine the border of a QTL block.

4. Characterization of QTL for multiple disease resistance

To uncover the resistance effects of MDR target regions, sets of F_{6:7}, F_{6:8} or F_{6:9} NILs contrasting for different target loci were developed from selected lines in HIFs. From 2007-2008, 15 sets of NILs differing at a total of 21 bins were evaluated for

Table 2.3 NLB QTL identified using HIF analysis. Loci for resistance to northern leaf blight (NLB QTL at bins 1.06, 5.03, 6.05, and 8.05-8.06) were identified in heterogeneous inbred families (HIFs) and near-isogenic lines (NILs) derived from the crosses of B73 x CML52 and S11 x DK888. Different disease parameters, including incubation period (IP; days post inoculation, dpi), lesion number (LN), diseased leaf area (DLA), and area under the disease progress curve (AUDPC), were evaluated. Trait values shown are relative allele effects, which are the differences on the least squares means between CML52 homozygous genotypes and B73 homozygous genotypes, or between DK888 homozygous genotypes and S11 homozygous genotypes at the locus. The significance level was determined by pairwise two-tailed Student's t test on the difference between least squares means (denoted as * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$; § non-significant after the Bonferroni correction; ns: non-significant; —: not applicable because the trait was not tested).

Cross of origin	QTL position		Mapping material		Resistance allele	NLB QTL in the back-ground	Allele effect (CML52 or DK888 homozygotes – B73 homozygotes)							
	Maize bin ^a	cM on IBM2n (Mb on physical) ^b	Source HIF (generation)	HIFs and NILs, sample size			R^2 ^c	IP (dpi)	LN (number)	DLA1 (%)	DLA2 (%)	DLA3 (%)	DLA4 (%)	AUDPC (%-day)
B73 x CML52	1.06 ^d	516.2-545.6 (170.5-177.8)	1889_1 (F ₃) ^d	F ₆ HIF, n=47	CML52	none	ns	ns	ns	—	—	—	—	—
				F ₇ HIF, n=47			0.28	1.5**	-14.5***	-7.2*	-8.7***	-11.4**	—	-244.1***
				8 F ₈ and 10 F ₉ NILs			0.66	ns	—	-7.5***	-10.3***	-9.5***	-11.0***	-282.0***
				3 F ₇ , 20 F ₈ and 13 F ₉ NILs			0.58	—	—	-5.1***	-11.3***	-13.8***	—	-480.8***
	6.05 ^d	267.9-395.0 (123.7-148.2)	1889_1 (F ₃) ^d	F ₆ HIF, n=47	CML52	none	0.16	3.1**	-14.6**	-4.5*	—	—	—	—
				F ₇ HIF, n=47			0.09	1.2**	-9.4*	-6.1*	-6.5*	-7.6*	—	-179.6*
				8 F ₈ and 10 F ₉ NILs			0.64	1.0***	—	-7.4**	-12.9***	-14.1***	-15.1***	-350.5***
				3 F ₇ , 20 F ₈ and 13 F ₉ NILs			0.53	—	—	-4.4***	-7.7***	-11.7***	—	-364.2***
			1889_3 (F ₃)	F ₆ HIF, n=98		none	0.12	ns	-14.4***	-7.0***	-5.4***	-6.0**	—	-160.9***
				6 F ₇ NILs			0.60	0.8*§	—	-11.5*	-20.5**	-17**	-16.3**	-464.5**
	5.03	140.0-223.9 (10.6-20.3)	1871_2 (F ₃)	F ₆ HIF, n=65	B73	1.06,	0.11	ns	8.2**	5.2*§	—	—	—	—
				F ₇ HIF, n=58		6.05	0.18	-0.4*§	2.7*	—	—	—	—	—
				8 F ₈ NILs			0.32	ns	—	3.5**	4.8*§	4.6*§	6.0**	126.9*
			1871_3 (F ₃)	F ₆ HIF, n=38			0.11	-2.9*§	ns	ns	—	—	—	—

Table 2.3 (Continued)

Cross of origin	QTL position		Mapping material		Resistance allele	NLB QTL in the back-ground	Allele effect (CML52 or DK888 homozygotes – B73 homozygotes)							
	Maize bin ^a	cM on IBM2n (Mb on physical) ^b	Source HIF (generation)	HIFs and NILs, sample size			<i>R</i> ^{2c}	IP (dpi)	LN (number)	DLA1 (%)	DLA2 (%)	DLA3 (%)	DLA4 (%)	AUDPC (%-day)
	1.07-	697.1-729.9	1873_2 (F ₅)	F ₆ HIF, n=91	CML52?	1.06,	0.10	2.0*	-6.0*§	-2.5*§	–	–	–	–
	1.08 ^c	(211.3-220.8)		F ₇ HIF, n=83		5.03,	0.07	ns	-10.7*§	-3.0*§	ns	-6.3***	–	-57.6*§
				4 F ₈ NILs		6.05	ns	–	ns	ns	ns	ns	ns	
	8.02-	123.9-299.9	1901_5 (F ₅)	F ₆ HIF, n=55	CML52?	1.06,	0.06	3.8**§	-3.3*§	–	–	–	–	–
	8.03 ^c	(12.6-101.6)		F ₇ HIF, n=53		6.05,	0.07	8.4*	-10.7*§	–	–	–	–	–
				5 F ₈ NILs		8.05-8.06	ns	ns	–	–	–	–	–	
S11 x DK888	8.05-	386.8-453.7	1851_1_2 (F ₆)	F ₇ HIF, n=53	DK888	unknown	0.23	5.7***	–	ns	–	–	–	–
	8.06	(136.2-156.0)		F ₈ HIF, n=96			0.61	6.5***	–	–	–	–	–	–
				2 F ₈ and 6 F ₉ NILs			0.93	-5.9***	–	-17.9***	-16.6***	-12.2***	–	-379.4***

^a The maize genome is composed of 100 designated bins. Each bin is a chromosomal segment between 2 core RFLP markers.

^b The map position was based on genetic map of intermated B73 x Mo17 population (version IBM 2008 neighbors) and the B73 physical map. The map interval of each QTL was assumed to extend halfway between two markers around each end of the QTL. Marker names are provided for the QTL mapped on a single marker basis (flanking markers were not tested).

^c Adjusted R -square, a ratio of mean squares instead of sum of squares (R -square), was used to account for different numbers of parameters in different models. The adjusted R -square for each QTL was retrieved from the model for AUDPC, or either LN or IP if AUDPC is not applicable.

^d Bins 1.06 and 6.05 were segregating in the same heterogeneous inbred families derived from the F₅ line 1889_1. The QTL effects of these two regions were verified in the NILs, with consideration of their correlation (details described in data analysis). Their allelic effects shown are the phenotypic effects of a given locus, with the other locus segregating in the background.

^e The effects of candidate NLB QTL at bins 1.07-1.08 and 8.02-8.03 were not validated in advanced NILs.

SLB, GLS, ALB, ASR, common rust, common smut, and Stewart's wilt. In view of the workload involved in testing multiple lines with multiple diseases, repeated second-year trials in 2008 were mainly conducted to confirm the positive results from 2007. If a target region was not significantly or marginally associated with any traits of a given disease, the corresponding set of NILs was excluded from further evaluation of the disease in the second year. However, to accurately determine the resistance spectra of the reliably expressed NLB QTL, NILs contrasting for bins 1.06, 6.05, and 8.05-8.06 were evaluated for responses to different diseases in at least two environments. In the trials of 2008, ~36% of the previously detected significant associations between locus and disease were not verified (eg. ASR resistance conferred by the CML52 allele at bin 7.04 was seen in 2007 but not 2008). This implied likely possibility of type II error for the loci not included in the second-year trials.

Among the four NLB QTL validated in the NILs (bins 1.06, 5.03, 6.05 from B73 x CML52, and bin 8.05-8.06 from S11 x DK888), bins 5.03 and 8.05-8.06 were not associated with resistance to any of the other diseases, suggesting that the resistance effects of B73 allele at bin 5.03 and DK888 allele at bin 8.05-8.06 are NLB-specific. Bins 1.06 and 6.05, on the other hand, were associated with resistance to more than one disease. The QTL at bins 1.06 and 6.05 were identified from the HIFs and NILs segregating for both loci. The QTL for NLB, ASR and/or Stewart's wilt were confidently declared, as their effects were observed to be significant, given the other locus was segregating or fixed (either homozygous for CML52 or B73 alleles) in the background. Epistatic interaction between NLB QTL at bins 1.06 and 6.05 was not observed.

Bin 1.06 was significantly associated with resistance to NLB and Stewart's wilt. The CML52 allele was effective in decreasing DLA for NLB by 7-10%, and PrimDLA caused by Stewart's wilt by ~28% (Table 2.4). The QTL position is in the

Table 2.4 Resistance spectrum of the QTL identified using HIF analysis. Near-isogenic lines (NILs) contrasting for targeted loci were derived from the heterogeneous inbred families (HIFs) of B73 x CML52 and S11 x DK888. Effects of the targeted loci were determined by evaluating sets of the NILs for resistance to a range of important maize diseases, including northern leaf blight (NLB), Stewart's wilt, anthracnose stalk rot (ASR), southern leaf blight (SLB), gray leaf spot (GLS), anthracnose leaf blight (ALB), common rust (Rust), and common smut (Smut). Two broader-spectrum QTL were observed: CML52 allele(s) at bin 1.06 was effective against NLB and Stewart's wilt, and CML52 allele(s) at bin 6.05 was effective against NLB and ASR. Trait values shown are relative allele effects, which are the differences on the least squares means between CML52 homozygous genotypes and B73 homozygous genotypes, or between DK888 homozygous genotypes and S11 homozygous genotypes at the locus. The significance level was determined by pairwise two-tailed Student's t test on the difference between least squares means (denoted as * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$; ns: non-significant; -: not applicable because the trait was not tested).

Cross of origin	QTL position		Mapping materials		Resistance allele	Allele effect (CML52 or DK888 homozygotes – B73 homozygotes)										
	Maize bin ^a	cM on IBM2n (Mb on physical) ^b	Source HIF (generation)	HIFs and NILs, sample size		NLB		Stewart's		ASR		SLB	GLS	ALB	Rust	Smut
						R ² ^c	AUDPC (%-day)	R ² ^c	Primary DLA (%)	R ² ^c	Dis-colored internodes (total %)					
B73 x	1.06 ^d	516.2-545.6	1889_1 (F ₅)	8 F ₈ and 10 F ₉ NILs	CML52	0.66	-282.0***	0.58	-28.2***	ns	ns	ns	ns	ns	ns	
CML52		(170.5-177.8)		3 F ₇ , 20 F ₈ and 13 F ₉ NILs		0.58	-480.8***	0.73	-15.1***	–	–	–	–	–	–	
	6.05 ^d	267.9-395.0	1889_1 (F ₅)	8 F ₈ and 10 F ₉ NILs	CML52	0.64	-350.5***		ns	0.22	-25.3***	ns	ns	ns	ns	
		(123.7-148.2)		3 F ₇ , 20 F ₈ and 13 F ₉ NILs		0.53	-364.2***		ns	–		ns	ns	ns	ns	
			1889_3 (F ₅)	6 F ₇ NILs		0.60	-464.5**		ns	ns		ns	ns	ns	ns	
	5.03	140.0-223.9	1871_2 (F ₅)	8 F ₈ NILs	B73	0.32	126.9*		–	ns		ns	ns	ns	ns	
		(10.6-20.3)														
S11 x	5.06	umc2216	1851_1_2 (F ₆)	F ₇ HIF, n=53	S11		ns		–	–		–	–	–	–	
DK888		(518.4)		2 F ₇ and 6 F ₈ NILs			–		–	0.57	23.6***	ns	ns	ns	ns	
				F ₈ HIF, n=139			–		–	0.05	14.3**	–	–	–	–	

Table 2.4 (Continued)

Cross of origin	QTL position		Mapping materials		Resistance allele	Allele effect (CML52 or DK888 homozygotes – B73 homozygotes)										
						NLB		Stewart's		ASR						
	Maize bin ^a	cM on IBM2n (Mb on physical) ^b	Source HIF (generation)	HIFs and NILs, sample size		<i>R</i> ² ^c	AUDPC (%-day)	<i>R</i> ² ^c	Primary DLA (%)	<i>R</i> ² ^c	Dis-colored internodes (total %)	SLB	GLS	ALB	Rust	Smut
	6.05	bnlg2249	1896_2_2 (F ₆)	2 F ₇ NILs	S11	–		–		0.42	80.9***	ns	ns	ns	ns	ns
		(278.0)		F ₈ HIF, n=129		–		–		0.04	41.4**	–	–	–	–	–
	6.05	bnlg1732	1896_1_3 (F ₆)	F ₇ HIF, n=74	none	ns		–		–		–	–	–	–	–
		(373.8)		2 F ₇ NILs		–		–		ns		ns	ns	ns	ns	ns
	8.05-8.06	386.8-453.7 (136.2-156.0)	1851_1_2 (F ₆)	2 F ₈ and 6 F ₉ NILs	DK888	0.93	-379.4***	ns		ns		ns	ns	ns	ns	ns

^a The maize genome is composed of 100 designated bins. Each bin is a chromosomal segment between 2 core RFLP markers.

^b The map position was based on genetic map of intermated B73 x Mo17 population (version IBM 2008 neighbors) and the B73 physical map. The map interval of each QTL was assumed to extend halfway between two markers around each end of the QTL. Marker names are provided for the QTL mapped on a single marker basis (flanking markers were not tested).

^c Adjusted *R*-square, a ratio of mean squares instead of sum of squares (*R*-square), was used to account for different numbers of parameters in different models.

^d Bins 1.06 and 6.05 were segregating in the same heterogeneous inbred families derived from the F₅ line 1889_1. The QTL effects of these two regions were verified in the NILs, with consideration of their correlation (details described in data analysis). Their allelic effects shown are the phenotypic effects of a given locus, with the other locus segregating in the background.

vicinity of *Sw1*, a dominant major gene locus for Stewart's wilt previously found in inbred line Ki14 (Ming et al. 1999; Pataky et al. 2008). The effect of bin 1.06 for resistance to both NLB and Stewart's wilt has also been observed from the NILs derived from the inbred line Tx303 crossed to B73 (details in Chapter 1). In this case, the Tx303 allele at bin 1.06 contributed moderate resistance to NLB and strong resistance to Stewart's wilt.

Bin 6.05 was significantly associated with resistance to NLB and ASR. The CML52 allele effectively decreased DLA by 7-15% for NLB (Table 2.4), and decreased total diseased area caused by ASR in the stalk by ~25%. Bin 6.05 is a novel ASR QTL, perhaps not previously mapped because QTL for ASR resistance have only been studied in two mapping populations: DE811ASR x DE811 and DE811ASR x LH132 (Jung, Weldekidan *et al.* 1994), from which an R gene underlying a major QTL at bin 4.07 has been cloned (Broglie et al. 2006). Two DK888-derived HIFs were segregating for either *bnlg2249* or *bnlg1732* in bin 6.05. The locus *bnlg1732* was not associated with resistance to NLB, ASR, or five other diseases. S11 allele at the locus *bnlg2249*, on the other hand, was associated with ASR resistance, while its resistance to NLB was not evaluated (Table 2.4). Although its effect for NLB resistance is unknown, the S11 allele at *bnlg2249* in bin 6.05 was more effective than the CML52 allele (spanning across *bnlg2249* and *bnlg1732*) in reducing ASR symptoms in maize stalks (reducing ~80 % and ~40% of total discolored internode area in NILs and HIFs, respectively). An ASR-specific QTL at bin 5.06 was also detected in the DK888-derived HIF. The S11 allele at bin 5.06 conferred resistance by significantly reducing total discolored internode area by ~24% in NILs and by ~14% in HIFs.

5. Phenotypic correlation for different diseases in RILs

Phenotypic correlation between different traits in a segregating population gives

indication of an underlying genetic mechanism in common between the two traits, which could be either linkage or pleiotropy. We looked at the Pearson correlation coefficients for each of the disease resistance traits (Table 2.5). As expected, there were significant correlations between NLB, SLB and GLS resistance and maturity. In general, later maturity results in less disease severity and this was seen in the RIL population. There was a significant positive correlation between NLB and GLS resistance, indicating co-localizing QTL or common QTL as indicated above. However, based on the QTL mapping results below, it was not clear that there were co-localizing QTL. Surprisingly, there was a negative correlation between SLB and ASR resistance. This is probably largely due to the closely linked QTL on chromosome 3 for the two diseases. For this region, resistance to SLB is conferred by CML52 while resistance to ASR is conferred by B73. The repulsion phase linkage of these two large-effect QTL likely produced the negative correlation.

Table 2.5 Phenotypic correlations between the traits of disease resistance and plant maturity. In the population of recombinant inbred lines (RILs) derived from B73 x CML52, Pearson correlation coefficients between the Best Linear Unbiased Predictions (BLUPs) values of days to anthesis (DTA), resistance to northern leaf blight (NLB), gray leaf spot (GLS), southern leaf blight (SLB), and anthracnose stalk rot (ASR) were computed. The significant correlation coefficients at P -value > 0.05 are shown.

	NLB	GLS	SLB	ASR	DTA
NLB		0.23			-0.19
GLS	0.23				-0.18
SLB				-0.17	-0.23
ASR			-0.17		
DTA	-0.19	-0.18	-0.23		

6. NLB, GLS, SLB, and ASR QTL identified in CML52 RILs

The profile of likelihood of odds ratio (LOD) scores for ICIM is shown in Fig. 2.3. As with any QTL mapping study, the decision to declare a significant QTL can be rather subjective. Permutation analysis for this data set provided the significance threshold of $\text{LOD} = 9$ for an experimental wide alpha of 0.05. At this threshold, only one locus would be declared significant. The low LOD values reflected the small sample size. The CML52 RILs dataset was part of a larger analysis based on the nested association mapping population of which the CML52 RILs population is a subset. Thus, responses to NLB, GLS, and ASR have been evaluated on a single replication per year for two, one, and two years, respectively. The third year of data for NLB and the second year of data for GLS, resulting from the trials in 2009, will be incorporated later. Because Type I error was not a great concern, a lower (arbitrary) threshold of $\text{LOD} = 3$ was temporarily used. At this low LOD level, the findings were quite consistent with the QTL identified using the HIF strategy. NLB QTL were identified at bin 1.06 (109.9-115.0 cM), bin 6.05 (61.8-72.3 cM), and bin 8.05-8.06 (76.1-82.3 cM). For SLB, four QTL were identified at bin 1.08 (147.9-151.8 cM), bin 2.03 (53.4-54.9 cM), bin 3.04 (53.4-54.7 cM), and bin 9.04 (47.2-53.1 cM). For ASR, one QTL was identified at bin 3.05 (63.8-64.1 cM). Using $\text{LOD} = 3$ as a significance criteria, no significant QTL were identified for GLS.

A QTL for NLB resistance was detected at bin 6.05. Co-localizing with this QTL was a peak for ASR, though the peak was only at a significance of $\text{LOD} = 1.9$. Looking only at the ICIM results, there is indication that this region could have MDR. However, based only on the QTL mapping in RILs, it would be difficult to conclude that there is evidence for MDR.

BIM gave a similar picture as likelihood interval mapping. With the Bayesian approach, it is difficult to place a significance threshold for the null hypothesis of no

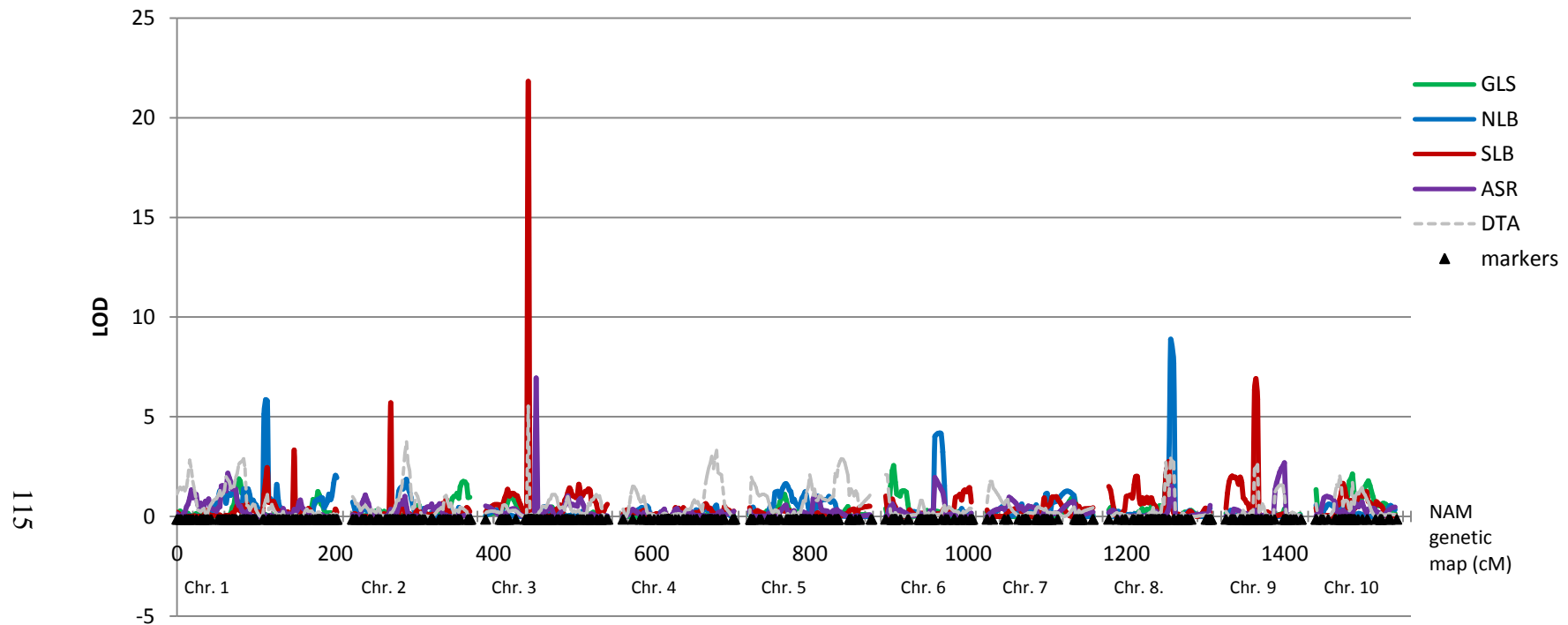


Figure 2.3 Likelihood profile of quantitative trait loci (QTL) detected by inclusive composite interval mapping (ICIM).

In the population of recombinant inbred lines (RILs) derived from the B73 x CML52 cross, loci conditioning resistance to northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS), and anthracnose stalk rot (ASR), as well as loci affecting days to anthesis (DTA), were identified using ICIM. The likelihood of odds ratio (LOD) scores for QTL across the 10 chromosomes of maize are shown. The triangles below each chromosomes are the positions of the 773 single nucleotide polymorphism (SNP) used in the study.

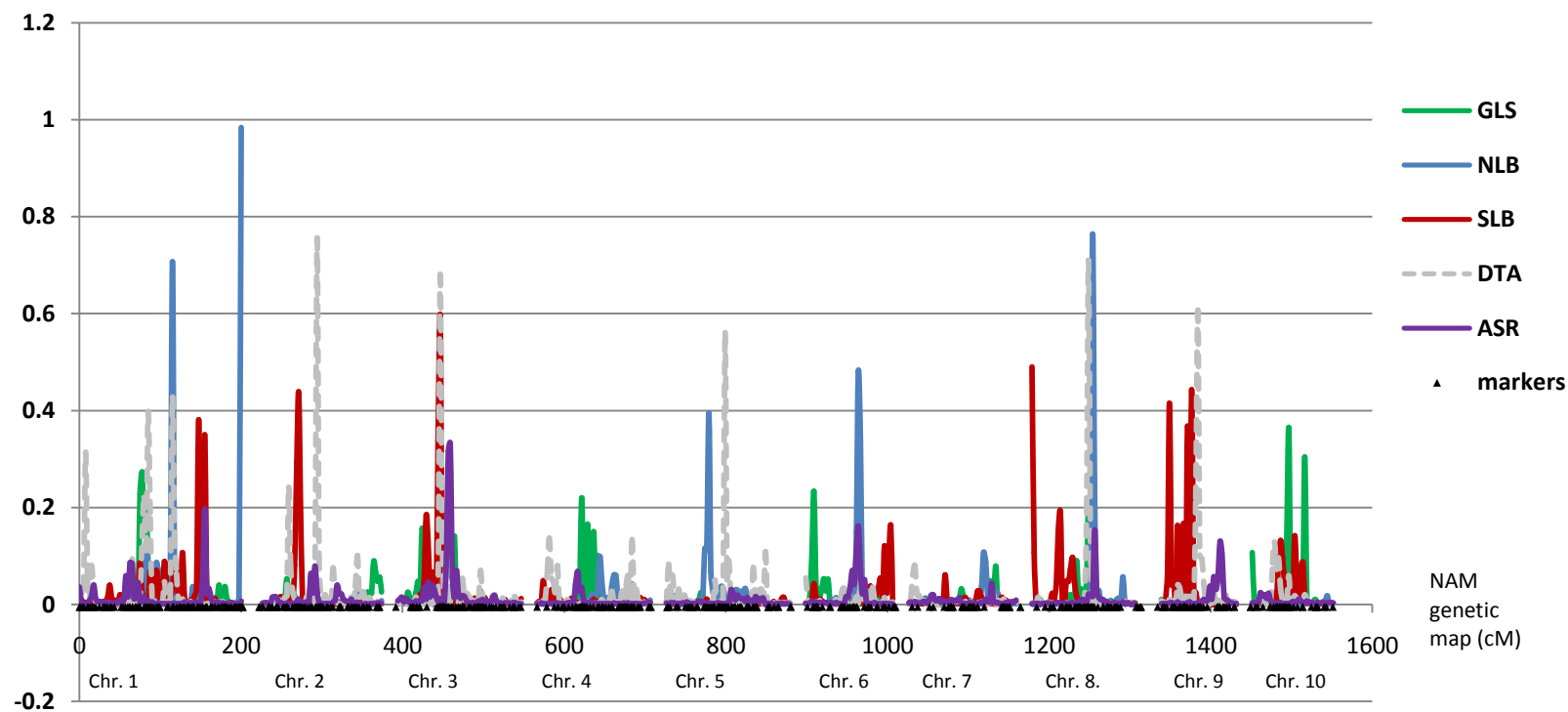


Figure 2.4 Likelihood profile of quantitative trait loci (QTL) detected by Bayesian interval mapping (BIM). In the population of recombinant inbred lines (RILs) derived from the B73 x CML52 cross, loci conditioning resistance to northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS), and anthracnose stalk rot (ASR), as well as loci affecting days to anthesis (DTA), were identified using BIM. The posterior probabilities of QTL across the 10 chromosomes of maize are shown. The triangles below each chromosomes are the positions of the 773 single nucleotide polymorphism (SNP) used in the study.

QTL effect for a given location. Fig. 2.4 shows the genome profile of the posterior QTL probably for each of the four diseases. As with ICIM, there was strong evidence of NLB QTL at bins 1.06 (115 cM), 6.05 (65 cM), and 8.05-8.06 (75 cM).

Additionally, there appeared to be evidence of NLB QTL at bin 1.08 (147.5-155.0 cM) as well as the very end of chromosome 1 at bin 1.12 (200 cM). Additional evidence of a QTL at bin 5.03 (52.5 cM) was also seen with BIM but not ICIM.

The BIM results for SLB resistance supported the conclusions from ICIM. With BIM, there was additional evidence of SLB QTL at the same positions in bins 1.08, 2.03, 3.04 and 9.04. On chromosome 9 there were multiple different peaks, including two distinct peaks at bins 9.02 (22.6 cM) and 9.04 (50.1 cM). The other lower peaks could also very well indicate multiple QTL, but they were not declared because of inconsistency in the ICIM result.

As with ICIM, there was strong evidence of QTL for ASR resistance at bin 3.05 (65 cM). As noted with ICIM, there appeared to be a QTL at bin 6.05 for ASR that co-localized with a QTL for NLB resistance. There was greater support for this QTL with BIM, and it again co-localized with the QTL for resistance from NLB.

Overall, in RIL-based mapping, QTL were declared if they were consistently detected with ICIM and BIM. Additional evidence from the HIF analysis was required for declaring QTL with minor effect. Disease QTL conformed to the criteria are listed in Table 2.6 with their genetic and physical map positions.

7. Pleiotropic QTL for disease resistance and flowering time

The late-maturing properties of CML52 and DK888 led to the question of whether the effects of disease QTL reflect indirect expression of flowering time QTL. To address this, the NILs and RILs were scored for days to anthesis (DTA) on a row basis and analyzed for potential QTL. In RIL-based mapping, both ICIM and BIM

Table 2.6 Disease QTL identified using recombinant inbred line (RIL) – based QTL mapping. Loci for resistance to northern leaf blight (NLB), southern leaf blight (SLB), gray leaf spot (GLS), and anthracnose stalk rot (ASR) were identified in a RIL population derived from the cross of B73 x CML52. The RIL population consisted of 196 F₆ lines was genotyped with 773 single nucleotide polymorphism (SNP) markers. QTL declared here are the ones being consistently detected using inclusive composite interval mapping (ICIM) and Bayesian interval mapping (BIM). The final QTL to be declared will be based on the analysis with the data from 2009 included. Loci for days to anthesis (DTA) were also mapped in the RILs, but only the DTA QTL co-localized with disease QTL are shown. Co-localized QTL identified using heterogeneous inbred family (HIF) analysis are also listed.

Maize bin ^a	Resistance or late-maturing allele	QTL identified in RILs using ICIM						QTL identified in RILs using BIM						Co-localized QTL identified in HIFs	
		QTL position ^b	Likelihood of odd					QTL peak ^b	Posterior probability					QTL position ^c	Resistance effect
		cM on NAM (Mb on Physical)	NLB	SLB	GLS	ASR	DTA	cM on NAM (Mb on Physical)	NLB	SLB	GLS	ASR	DTA	cM on IBM2n (Mb on Physical)	
1.06	CML52	109.9-115.0 (184.6-188.8)	5.9				1.1	115.0 (188.7)	0.71				0.43	516.2-545.6 (170.5-177.8) ^d	NLB, Stewart's wilt
1.08	CML52	147.9-151.8 (240.3-248.3)	1.5	3.3				147.5-155.0 (242.3-245.6)	0.34	0.38				–	–
1.12	B73	196.5-202.2 (284.4-287.5)	2.1					200.0 (285.6-286.7)	0.98					–	–
2.03	CML52	53.4-54.9 (21.5-22.3)		5.7				51.4 (17.4-20.7)		0.44				–	–
3.04	CML52	53.4-54.7 (26.7-30.6)		21.8			5.5	52.5 (20.4-26.8)		0.59			0.68	–	–
3.05	B73	63.8-64.1 (129-131.9)				6.9		65.0 (121.9-137.4)				0.33		–	–
5.03	B73	37.9-53.6 (12.4-21.9)	1.6					52.5 (19.1-21.8)	0.40					140.0-223.9 (10.6-20.3)	NLB
6.05	CML52	61.8-72.3	4.2			2.0		65.0	0.48			0.16		267.9-395.0	NLB, ASR

Table 2.6 (Continued)

Maize bin ^a	Resistance or late-maturing allele	QTL identified in RILs using ICIM						QTL identified in RILs using BIM						Co-localized QTL identified in HIFs	
		QTL position ^b	Likelihood of odd				cM on NAM (Mb on Physical)	QTL peak ^b	Posterior probability					QTL position ^c	Resistance effect
		cM on NAM (Mb on Physical)	NLB	SLB	GLS	ASR		cM on NAM (Mb on Physical)	NLB	SLB	GLS	ASR	DTA	cM on IBM2n (Mb on Physical)	
		(141.9-148.6)						(141.9-147.4)						(123.7-148.2)	
8.05-8.06	CML52	76.1-82.3 (139.3-148.4)	8.9			1.5	2.9	75.0 (137.6-139.3)	0.76			0.15	0.71 ^e	386.8-453.7 (136.2-156.0)	NLB (DK888 allele)
9.02	CML52	14.6-34.6 (13.9-27.5)		2.02				22.6 (17.6-19.3)		0.42				–	–
9.04	CML52	47.2-53.1 (53-92.8)		6.9			2.6	50.1 (72.2-76.3)		0.44			0.61 ^e	–	–

^a The maize genome is composed of 100 designated bins. Each bin is a chromosomal segment between 2 core RFLP markers.

^b For QTL identified in RILs, the map position was based on genetic map of the nested association mapping (NAM) population and the B73 physical map. In ICIM, the map interval of each QTL was estimated based on the markers flanking the QTL peak (LOD > 1.5). In BIM, the location of each QTL peak is shown (with the physical positions based on the flanking makers).

^c For QTL identified in HIFs, the map position was based on genetic map of intermated B73 x Mo17 population (version IBM 2008 neighbors) and the B73 physical map. The map interval of each QTL was assumed to extend halfway between two markers around each end of the QTL.

^d The QTL position in the HIFs did not overlap with the position estimated using the RIL-based analysis. The borders of the QTL will be checked in the HIFs with more polymorphic markers.

^e Using BIM, the DTA QTL were mapped to loci closely linked to (but not coincided with) the disease QTL. The DTA QTL were localized to bin 8.04-8.05 at 64.1-66.9 cM (111.0-116.0 Mb), and bin 9.04 at 57.6 cM (88.2-89.1 Mb).

detected several QTL peaks for DTA, with the majority showing minor effects (Fig. 2.3 and 2.4). This corresponded to the finding that the genetic architecture of flowering time in maize is mainly controlled by small additive QTL (Buckler et al. 2009). Four DTA QTL at bins 1.06, 3.04, 8.04-8.06, and 9.04 coincided with either NLB or SLB QTL (Table 2.6). While the DTA QTL at bins 8.04-8.06 and 9.04 were well co-localized with the disease QTL using ICIM, they were mapped to loci closely linked but not overlapping positions using BIM. Additional DTA QTL at bins 2.05 and 5.04 were also identified with BIM (DTA QTL at bin 1.01: 7.5 cM, 44.3-52.9 Mb, posterior probability = 0.31; DTA QTL at bin 1.04-1.05: 85 cM, 73.8-78.6 Mb, posterior probability = 0.40; DTA QTL at bin 2.05: 79.8 cM, 140.3 Mb, posterior probability = 0.76; DTA QTL at bin 5.04: 72.5 cM, 144.5 Mb, posterior probability = 0.56). Fine-mapping and further examination of the QTL affecting disease resistance and flowering time will be needed for distinguishing linkage from pleiotropy for these loci. For several of the disease QTL, no association with maturity was detected.

In the HIF analysis, although variation for flowering time was sometimes observed among NILs within a set, none of the targeted loci were found to affect DTA, suggesting that the resistance conferred by the identified disease QTL was not significantly associated with flowering time. The results from CML52-derived NILs largely agreed with the results from RIL-based mapping, except for the identification of co-localized DTA and NLB QTL at bin 1.06 in RILs but not NILs. No DTA effect was detected in the NILs segregating for bin 1.06, perhaps due to genetic background effect, or because the segregating region did not contain gene(s) affecting flowering time (linkage rather than pleiotropy for the DTA and NLB QTL at bin 1.06).

DISCUSSION

1. Disease QTL identified using HIF- and RIL-based approaches

To discover maize loci conditioning resistance to single as well as multiple diseases, HIF- and RIL-based approaches were applied for QTL mapping using genetic materials derived from the broadly resistant maize lines CML52 and/or DK888. Both approaches respectively identified several QTL, and the parallel studies provided additional evidence for validation of the detected QTL, particularly the ones with minor effects.

The HIF-based QTL approach aimed to capture and characterize resistance alleles at loci associated with previously identified major genes and disease QTL. The selected CML52- and DK888-derived HIFs were among the advanced inbred populations available at the inception of the study. We applied 73 SSR markers to target 39% of the maize genome, focusing on the regions associated with clusters of previously reported disease genes and QTL. Our strategy was to first identify QTL for resistance to NLB (our primary disease of focus) in segregating HIFs, to generate sets of NILs contrasting for the candidate NLB QTL, and to use the derived NILs for NLB QTL validation and characterization of resistance spectrum. Using HIF analysis, we detected and validated four NLB QTL at bins 1.06, 5.03, 6.05 and 8.05-8.06, two ASR QTL at bins 5.06 and 6.05, and one Stewart's wilt QTL at bin 1.06. The average size of the identified QTL was 85 cM on the IBM2n map, which is smaller than the average disease QTL size [107 cM on the IBM2n map (Wisser et al. 2006)]. The precision of QTL locations was improved with increased marker density (66 additional markers) surrounding the QTL regions.

Using the RIL-based approach, QTL peaks were identified for NLB at bins 1.06, 1.08, 1.12, 5.03, 6.05 and 8.05-8.06, for SLB at bins 1.08, 2.03, 3.04 and 9.03, and for

ASR at bin 3.05 and 6.05. The statistical validity of these peaks can be questioned; the final QTL to be declared as a result of this analysis will not be determined until the data from the trials in 2009 are analyzed. Different statistical methods for QTL mapping have been developed to improve the genome-wide genetic analysis of complex traits. While ICIM and BIM take different statistical approaches to identification of QTL, it is expected that there should be concordance in the results for 'real' QTL. We found in our study this was generally the case, especially for QTL that were validated in the HIFs. Since the RILs utilized in the study were genotyped with a high density of SNP markers, the QTL resolution was significantly increased. The average size of the identified QTL is 4.5 cM on the NAM map, and 18.6 Mb on the physical map. The high-precision mapping successfully distinguished the SLB QTL and ASR QTL on chromosome 3.

Mapping results from the parallel HIF- and RIL-based studies were used for cross-validation. QTL identified in HIFs/NILs largely conformed to the QTL mapped in RILs. Most of the genetic regions that have been investigated in the CML52 HIFs/NILs (27 bins as a total) did not show significant effects for resistance to NLB, SLB, GLS or ASR, which was consistent with their lack of phenotypic effects in the RILs. HIF- and RIL-based approaches detected co-localized NLB QTL at bins 1.06, 5.03 and 6.05, and co-localized ASR QTL at bin 6.05 (QTL were considered co-localized if they are overlapping or nearly overlapping). The effects conferred by the NLB QTL at bin 5.03 and ASR QTL at bin 6.05 appeared to be moderate. In RIL-based mapping, the two minor QTL were more clearly identified using BIM, but were only suggestively detected by ICIM method with LOD values well below the threshold. It is also noteworthy that five large-effect QTL for resistance, including NLB QTL at bin 8.05-8.06, SLB QTL at 2.03, 3.04 and 9.03, and ASR QTL at bin 3.05, as well as two minor-effect NLB QTL at bins 1.08 and 1.12, and one minor-

effect SLB QTL at bin 1.08 (only detected with BIM), were mapped in RILs but not HIFs/NILs. This is simply because the genetic regions were not targeted by the markers utilized, or were fixed in all the available HIFs. (Note that the region of bin 1.08 tested in CML52 HIFs was ~20 Mb distant from the NLB/SLB QTL identified at bin 1.08 in the RILs. As a result, the NLB QTL at bin 1.08 was not captured or evaluated in HIFs.)

2. HIF-based QTL analysis: considerations and lessons learned

The use of RILs is an efficient way of getting a whole-genome QTL map at reasonable resolution, especially when populations with high-density marker data are available. In the present study, RIL-based mapping detected all the QTL identified in HIFs, as well as several additional QTL. The objective of this study, however, was not to identify all QTL in a given genotype, but rather to test the hypothesis that selected loci from selected genotypes condition MDR. Relevant RIL populations were not available at the inception of this study, whereas we had access to HIFs suitable for the extraction of NIL pairs. We have demonstrated that HIF-derived NILs can be used for QTL mapping, particularly when, as in this study, partially-inbred materials are available and certain loci are to be targeted.

The efficiency of HIFs in this study was the use for analysis of multiple diseases at specific loci associated with MDR. Even if RIL populations had been available at the inception of the study, it would not have been feasible to analyze them for multiple diseases in a reasonable period of time and field space. Our HIF strategy, as modified from the methodology of Tuinstra *et al.* (1997), involved selecting QTL for the primary focus of disease (NLB), generating NILs for those QTL, and testing them for response to other diseases. To be able to focus on the regions for resistance to NLB, we conducted individual trait-marker analysis in earlier generations of HIFs,

which may cause higher probability of type II error due to the evaluation of individual plants with less homogeneous genetic backgrounds. This problem was addressed by confirming the initially identified candidate QTL in another HIF(s) and/or advanced NILs. Along the way, selfed seeds were obtained from a large proportion of genotyped lines within selected HIFs, which became useful as sources of NILs and segregating populations suitable for subsequent QTL characterization and fine-mapping. As a result, the QTL at bins 1.06 and 6.05 were confirmed to confer broader-spectrum resistance, and the NLB-specific QTL at bin 8.05-8.06 was delimited to a region of 460 kb in a follow-up study (details in Chapter 3).

While a HIF-based approach was successfully utilized, certain weaknesses should be acknowledged. The outcome of HIF-based analysis is determined by the genetic structure of the starting materials. Starting with limited number of F_5 or F_6 families, HIFs segregating for a given locus may or may not be available. While the large-effect QTL in targeted regions were clearly identified using HIFs, some QTL identified only in the RILs either because they were not targeted or because of the nature of the starting materials: they were fixed in the selected HIFs or were captured in NIL pairs with very resistant backgrounds. In this study, it is difficult to determine the relative detection power of HIF- and RIL-based mapping by comparing allelic effects of a given QTL resulting from the two approaches. Evaluations of HIFs and RILs were conducted by different researchers in different environments. The magnitudes of QTL effects as well as the proportion of phenotypic variation that are explained by QTL have proven to be highly vulnerable to different people's ratings (J. Poland, pers. comm.). Using simulated data, Kaeppler (Kaeppler 1997) showed that QTL mapping using RILs generally (and sometimes substantially) provided better power over the use of backcross-derived NILs, despite the higher precision phenotyping in the NILs. Since the HIF-based approach is conducted on the basis of

contrasting effects of pairs of NILs, it may provide similar power as the conventional NIL-based approach, and therefore less power than RIL-based approach.

The major weakness of the HIF-based approach to NIL development is the effect of diverse backgrounds in selected HIFs and derived NIL pairs. In contrast to backcross-derived NILs, which are in the background of the recurrent parent, HIF-derived NILs have genetic constitutions recombined from two parental genomes. The randomly recombined background of HIF-derived NILs may or may not be appropriate for QTL expression. The observed effectiveness of a QTL can be masked by major-effect QTL (Eshed and Zamir 1995; Keurentjes et al. 2007) or affected by epistatic QTL (Njiti et al. 1998; Szalma et al. 2005) in the unlinked region(s) of the genome. In this study, QTL effects were masked by genetic backgrounds in two NIL pairs. All the QTL detected using the HIF-based approach were identified in HIFs/NILs exhibiting low to moderate levels of resistance. By contrast, the minor-effect NLB QTL at bin 5.03 was not found to be effective in a HIF carrying resistance alleles at three larger-effect NLB QTL. The masking or epistatic effect caused by genetic background has been observed in other studies, in which some candidate QTL were validated in the NILs derived from some but not all the chosen HIFs (Pumphrey et al. 2007; Tuinstra et al. 1997).

The fact that disease QTL effects can be highly dependent on genetic background, and the minor QTL effects can be masked by major QTL effects has implications for the value of marker-assisted selection (MAS) in resistance breeding programs. Use of MAS has been proposed (and demonstrated in some cases) to be more efficient than conventional phenotypic selection for traits that are difficult to manage (Flint-Garcia et al. 2003; Ribaut and Ragot 2007; Xu and Crouch 2008; Yousef and Juvik 2001). This refers to the penetrance of the target loci and the costs associated with phenotyping. As a complex trait, disease resistance is controlled by multiple loci

affecting diverse defense mechanisms. Pyramiding favorable alleles for multiple QTL conditioning resistance to a single disease and/or various diseases is expected to result in resistance that is more durable. However, in practice, phenotypic selection alone may not be effective for combining desirable alleles for multiple QTL, as some QTL effects would be undetectable in certain working backgrounds. MAS, on the other hand, is relatively attractive because of its potential of tracking and pyramiding favorable chromosomal segments regardless of the manifested phenotypes.

3. Implications of multiple disease resistance (MDR)

Because of our interest in the phenomenon of MDR, we inoculated a diverse set of pathogen species onto a small set of maize genotypes reputed to possess MDR, as well as on a large set of their near-isogenic derivatives, targeting chromosomal regions previously shown to be associated with MDR. MDR was observed in the study at the level of genotype and co-localized QTL. Among the genotypes used as sources of alleles, the hybrid DK888 exhibited superior resistance to all the eight diseases under testing; the tropical inbred line CML52 showed superior to moderate levels of resistance to all the tested fungal leaf diseases, including NLB, SLB, GLS, ALB, and common rust, and the bacterial disease Stewart's wilt; the elite inbred line B73 showed great resistance only to common rust and common smut, which are caused by biotrophic fungi. Using HIF- and/or RIL-based QTL analyses, we revealed that CML52 alleles at bins 1.06, 1.08, and 6.05 confer resistance to different diseases. Bin 1.06 is associated with co-localized NLB QTL and Stewart's wilt QTL, bin 1.08 is associated with co-localized NLB QTL and SLB QTL, and bin 6.05 is associated with co-localized NLB QTL and ASR QTL. Identification of these QTL confirmed the hypothesized existence of chromosomal segments conditioning MDR in broadly resistant genotypes.

Genotypes with MDR characteristics and genomic regions contributing MDR have been recognized for a range of plants. MDR phenotype could be due to pyramiding or linkage of genes with disease-specific effects, or to the presence of gene(s) with broad-spectrum effects. In general, disease-specific resistance is controlled by resistance (R) genes, which encode proteins that can recognize specific pathogen effectors and trigger rapid induction of the hypersensitive response and a series of defense reactions (Jones and Dangl 2006). Genes with pleiotropic effects, on the other hand, may be key regulatory genes controlling the recognition or signaling of non-host resistance, basal resistance, and systemic acquired resistance [eg. *mlo* (Buschges et al. 1997), *npr1* (Cao et al. 1998), and *Lr34* (Krattinger et al. 2009)]. They may also be defense response (DR) genes functioning as the downstream components of a variety of defense mechanisms [eg. pathogenesis-related (PR) genes (Edreva 2005)]. In addition, the specificity of resistance may be associated with temporary and spatial induction of antimicrobial structures or compounds, and the spectrum of these defense responses. This type of broad-spectrum resistance, controlled by a single pleiotropic gene or multiple genes, could affect pathogens with similar mode of pathogenesis.

In the study, we examined maize resistance to eight pathogens with diverse yet partially overlapping lifestyles. Resistance against the pathogens sharing certain stages of pathogenesis implicates defense mechanisms targeting certain aspects of plant-microbe interaction. The pathogens studied here can be divided into three main categories (Oliver and Ipcho 2004): (1) biotrophs that derive energy from living cells (*P. sorghi* for common rust, *U. maydis* for common smut, and *P. stewartii* for Stewart's wilt), (2) necrotrophs that derive energy from killed cells (*C. heterostrophus* for SLB), and (3) hemibiotrophs that develop initially as biotrophs and later as destructive necrotrophs (*S. turcica* for NLB, *C. zea-maydis* or *C. zeina* for GLS, and *C. graminicola* for ALB and ASR). Distinct and common defense mechanisms for

biotrophic versus necrotrophic pathogens have been well-documented (Glazebrook 2005; Koornneef and Pieterse 2008; Thaler et al. 2004). In our repeated field and greenhouse trials, B73 showed moderate susceptibility to many diseases but high levels of resistance to common rust and common smut, suggesting that it carries some recognition gene(s) and/or downstream defense mechanisms against obligate biotrophic fungi. Likewise, in the RIL population, we observed correlation between resistance to NLB and GLS, both caused by hemibiotrophic fungi, but independence of resistance to SLB, caused by the necrotrophic fungus. Consistent correlation relationships among resistance to the three diseases has been observed, with maturity and population structure taken into account, in a panel of 253 diverse maize lines (R. Wisser, J. Kolkman, R. Nelson and P. Balint-Kurti, pers. comm.). These observations suggested the existence of resistance components specifically against biotrophic, hemibiotrophic or necrotrophic pathogens.

The pathogens can also be classified based on their natures of infecting and colonizing different plant tissues. The unique and shared characteristics among these pathogens may in part reflect the possibility of finding specific types of defense mechanisms underlying the QTL at bin 1.06, 1.08 and 6.05. As mentioned above, the MDR observed for these chromosomal segments may result from linkage of disease-specific loci or from gene(s) affecting more than one pathogen species. If assuming the latter, we can consider what types of function could lead to the observed patterns of resistance.

Bin 1.06 is associated with resistance to two diseases that cause wilting lesions by pathogen growth within the xylem. Although one disease is fungal (NLB, caused by *S. turcica*) and the other is bacterial (Stewart's wilt, caused by *P. stewartii*), both might be affected by defense mechanisms that either prevent pathogen invasion or eliminate pathogen growth and dissemination in the xylem lumen. In rice, a peroxidase has been

implicated in the vascular defense response against the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*. Induction and accumulation of a peroxidase, PO-C1, in the xylem parenchyma, vessel walls and lumen is associated with the rapid thickening of the xylem secondary wall and the generation of antimicrobial reactive oxygen species (Hilaire et al. 2001). This and/or other defense mechanisms could function in the maize vascular system. A large number of peroxidases as well as other defense-related proteins, such as chitinase, beta-glucanase, and polygalacturonase inhibitor (PGIP), have been characterized in the maize xylem sap. The antifungal activity of the sap proteins has also been confirmed (Alvarez et al. 2006).

Bin 1.08 was found to be associated with resistance to SLB and NLB, leaf diseases caused by the necrotrophic fungus *C. heterostrophus* and the hemibiotrophic fungus *S. turcica*, respectively. The two pathogens have distinct early stages of pathogenesis. *C. heterostrophus* penetrates through the junctions between epidermal cells, and grows intercellularly in the leaf. *S. turcica*, on the other hand, penetrates the epidermal cell, and grows intracellularly and intercellularly from primary infected cell to surrounding chlorenchyma cells. During the later stages of pathogenesis, while *C. heterostrophus* parasitizes the chlorenchyma but does not invade the vasculature, *S. turcica* can invade into the xylem, efficiently proceed through it, and grow out from the xylem vessels into the surrounding bundle sheath and chlorenchyma. The development of both pathogens in maize leaves might be restricted by defense mechanisms that constrain their destructive growth in the chlorenchyma. It is difficult, however, to identify a candidate mechanism that would fail to protect against other leaf diseases, such as GLS and ALB, for which we did not observe resistance associated with bin 1.08.

The CML52 allele(s) at bin 6.05 were associated with resistance to NLB and ASR, while the same chromosomal segment from S11 was associated with resistance to ASR but not NLB or other diseases. NLB and ASR occur in distinct tissues (leaf versus

stalk tissues). The two causal organisms, *S. turcica* and *C. graminicola*, both hemibiotrophic fungi, share similar modes of initial colonization. Their early development involves biotrophic interactions with primary infected epidermal cells, and the subsequent intercellular hyphal growth and colonization of chlorenchyma or parenchyma cells. (*C. graminicola* directly colonizes the parenchyma cells if infecting through insect or artificial wounding.) Different means of progression are used by *S. turcica* and *C. graminicola*: the former grows aggressively in the xylem vessels then spreads into the neighboring chlorenchyma cells, while the later colonizes the mostly nonliving fiber cells associated with the vascular bundles and rind, then breaks into the adjacent parenchyma cells (Venard and Vaillancourt 2007a). For a single defense mechanism to work against both pathogens, it would have to function in both leaf chlorenchyma and stalk parenchyma, before the pathogen enters into the respective tube structures, or after the pathogen emerges to consume the neighboring tissues. There is little reason to expect that resistance expressed in the leaf and stalk tissues would be controlled by the same gene(s). Leaf and stalk resistance to *C. graminicola* (ALB and ASR, respectively) have been found to be non-correlated (Lim and White 1978; Zuber et al. 1981). Similarly, no correlation was observed between ASR and ALB resistance among the HIFs in the present study, and none of our identified ASR QTL showed ALB effects. In maize, the stalk versus leaf-specific resistance has been suggested based on the extremely different infection and colonization efficiencies of a closely related species *C. sublineolum* on the unwounded stalk and leaf tissues (Venard and Vaillancourt 2007b). It has also been shown that ASR resistance in a maize cultivar was not effective against *C. graminicola* in the root (Sukno et al. 2008). However, although tissue-dependent resistance may exist, wound healing mechanisms should explain part of the distinct defense responses in the stalk (Bergstrom and Nicholson 1999), as *C. graminicola* is mostly introduced into the stalk through

wounding either in the nature or in the resistance evaluation trials. It is thus likely that resistance to NLB and ASR are conditioned by linked genes in bin 6.05.

CONCLUSION

Several disease-specific QTL and three MDR QTL were identified and validated using HIF-based targeted QTL analysis and classical RIL-based QTL mapping. While the value of the disease QTL consensus map to MDR QTL prediction is still unclear, detection of chromosomal segments conditioning resistance to more than one disease reflected the generally observed clustering of disease QTL in plants. Overall, the study allowed empirical comparisons for the advantages and limitations of using HIFs and RILs for different application purposes. Evaluations of a range of pathogens causing important diseases, on the other hand, provided a snapshot of potential defense mechanisms contributing MDR in maize. QTL resolution in the study is higher than conventional QTL mapping, but is not high enough for determining candidate genes affecting resistance. Fine-mapping and positional cloning is underway to resolve the complex genetic mechanisms.

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CHAPTER 3

CHARACTERIZATION AND FINE-MAPPING OF A RESISTANCE LOCUS FOR NORTHERN LEAF BLIGHT IN MAIZE BIN 8.06⁴

ABSTRACT

As part of a larger effort to capture diverse alleles at a set of loci associated with disease resistance in maize, DK888, a variety known to possess resistance to multiple diseases, was used as a donor in constructing near-isogenic lines (NILs). A NIL pair contrasting for resistance to northern leaf blight (NLB), caused by *Setosphaeria turcica*, was identified and associated with bin 8.06. This region of the maize genome had been associated in previous studies with both qualitative and quantitative resistance to NLB. In addition, bins 8.05-8.06 had been associated with quantitative resistance to several other diseases, as well as resistance gene analogs and defense response gene homologs. To test the hypothesis that the DK888 allele at bin 8.06 (designated *qNLB8.06_{DK888}*) conditions the broad-spectrum quantitative resistance characteristic of the donor, the NILs were evaluated with a range of maize pathogens and different races of *S. turcica*. The results revealed that *qNLB8.06_{DK888}* confers race-specific resistance exclusively to NLB. Allelism analysis suggested that *qNLB8.06_{DK888}* is identical, allelic, or closely linked and functionally related to *Ht2*. The resistance conditioned by *qNLB8.06* was incompletely dominant and varied in effectiveness depending upon allele and/or genetic background. High-resolution breakpoint analysis, using ~2800 individuals in F₉/F₁₀ heterogeneous inbred families

⁴ Tiffany Jamann participated in genotypic and phenotypic data collection since Oct 2008. Joy Longfellow participated in genotypic and phenotypic data collection in 2006.

and 98 F₁₀/F₁₁ fixed lines carrying various recombinant events, delimited *qNLB8.06_{DK888}* to a region of ~0.46 Mb, spanning 143.92-144.38 Mb on the B73 physical map. Three compelling candidate genes were identified in this region. Isolation of the gene(s) will contribute to a better understanding of this complex locus.

INTRODUCTION

Plants have evolved diverse mechanisms to combat pathogens. Some defense mechanisms condition complete resistance, while others provide intermediate forms of resistance. Mechanisms of complete resistance include R-genes and non-host resistance. R-gene mediated resistance has often proven ephemeral, while quantitative resistance has generally been recognized as moderately effective, race non-specific and durable. Quantitative disease resistance (QDR) has therefore been more widely utilized in resistance breeding programs. QDR may, however, be conditioned by diverse mechanisms, and may vary in performance (Poland et al. 2009a). When QDR is conditioned by genes involved in recognition of evolutionarily labile pathogen effectors, it is likely to be both race-specific and non-durable. An understanding of the pathogen- and race-specificity of a locus is more likely to provide predictive power regarding the durability of resistance than its quantitative effect alone.

A range of mechanisms have been associated with QDR, some of which are broader in spectrum and more durable than others. Broad-spectrum resistance has commonly been divided into two classes: (1) resistance effective against all known variants of a given pathogen (“race non-specific resistance”) and (2) resistance effective against more than one pathogen (“multiple disease resistance”). Some major resistance genes have been observed to confer moderate levels of either race-

specific [eg. *Rp1* in maize (Smith and Hulbert 2005)] or race-nonspecific resistance [eg. *RB* in potato (Song et al. 2003)]. Despite the lower selection pressure caused by partial resistance, virulent pathogen races can gradually evolve to overcome the surveillance of partial resistance genes. For example, a recently emerged wheat stem rust strain, Ug99, is capable of overcoming *Sr31*, a resistance gene that had been extensively used worldwide and that had shown race-nonspecific and effective resistance for more than 30 years (Ayliffe et al. 2008). Race-nonspecific QDR has also been shown to be associated with mechanisms other than R-genes. For example, the *Yr36* gene in wheat contains domains similar to the proteins involved in the signaling of non-R-gene mediated defenses, including programmed cell death and innate immune response (Fu et al. 2009). In rice, the recessive allele of a susceptibility gene *Pi21*, encoding a proline-rich protein with putative heavy-metal binding and protein-protein interaction motifs, contributes resistance to blast disease (Fukuoka et al. 2009). The resistance of these non-R-genes has thus far been stable. Disease non-specific QDR have been found to be controlled by genes involved in basal resistance, systemic acquired resistance, and defense signaling pathways [eg. *RPW8.1* and *RPW8.2* in *Arabidopsis* (Wang et al. 2007c); *npr1* in *Arabidopsis* (Cao et al. 1998)]. Agriculturally-important genes of this type, including *Lr34* in wheat (Krattinger et al. 2009) and *mlo* in barley (Buschges et al. 1997), have been shown to confer durable resistance. Available lines of evidence imply that durability of resistance is associated with non-specificity, as well as non-gene-for-gene recognition in mechanism and incomplete phenotype. Nevertheless, the ambiguity associated with the effectiveness and spectra of defense mechanisms complicates breeding for disease resistance.

A large number of studies have been conducted to map R-genes, resistance gene analogs (RGAs), and loci conditioning QDR (quantitative trait loci for disease, or

disease QTL) in plants. Current knowledge in the genetic architecture of disease resistance, as inferred from overview of previous reports, may provide some insights on the types of resistance associated with different genetic loci, which may in turn have implications for the likely performance of genes at these loci. It has been widely noted that R-genes and disease QTL are not randomly distributed across the genome. Apparent clusters of QTL for different diseases have been observed in rice (Wisser et al. 2005), maize (Wisser et al. 2006), barley (Williams 2003), and other plants. The coincidence of defense-related genes and/or QTL for multiple pathogens in certain chromosomal segments has led to the hypothesis that these chromosomal regions are associated with broad-spectrum resistance that could be durable. Likewise, in a range of plant pathosystems, major genes and/or QTL affecting a given disease has been found to overlap. Association of major genes along with QTL [eg. *rhm* and QTL for southern leaf blight, and *Rp3* and QTL for common rust in maize (Wisser et al. 2006)] may reflect the differential major and minor effects conferred by allelic variants of identical gene(s) (Robertson 1989; Welz and Geiger 2000), or the differential expression of resistance in various genetic backgrounds or environments. At a gene level, complex clustering of homologous or non-homologous R-genes [eg. *Pi5* in rice (Lee et al. 2009) and *Rp1-D* in maize (Collins et al. 1999)] has been suggested as a genetic hallmark of rapid evolution of R-genes and race specificities (Hulbert et al. 2001; McDowell and Simon 2006).

Chromosomal regions associated with previously reported R-genes, RGAs, and disease QTL can be sources of genes conditioning diverse forms of resistance. However, due to the limitations of QTL analysis, such as low precision of QTL locations and allelic sampling in different studies (Wisser et al. 2006), the implication for resistance specificity should be used with caution. For a given allele at a disease QTL hotspot region, detailed evaluation will be required in order to clearly determine

whether it confers broad-spectrum or disease-specific resistance. Race-specificity of disease QTL, particularly for ones that co-localize with known R-genes, needs to be clarified prior to practical application. This is to prevent the deployment of disease QTL under the misleading assumption of QDR conferring non-specific and more durable protection for crops. Expectations for the long-term performance of a disease QTL can be more realistic if its underlying genetic basis is more fully explored. For instance, knowing whether the broad-spectrum phenotypes is conditioned by a pleiotropic gene(s), a cluster of defense-related genes, or the linkage of diverse R-genes, is valuable in designing combinations of favorable alleles of resistance genes in crop breeding programs.

In the maize genome, among the regions that may harbor genes involving diverse defense pathways, the 5-6th segment of chromosome 8 (bin 8.05-8.06) can be viewed as one of the most complex, important, and interesting. Bin 8.05-8.06 is known to be associated with QTL for resistance to various diseases, resistance gene analogs (RGAs), and defense response gene homologs (DRHs). Co-localized QTL were mapped in different populations for resistance to northern leaf blight (NLB) (Schechert et al. 1999; Welz et al. 1999a; Welz et al. 1999b), southern leaf blight (also known as southern corn leaf blight, hereafter referred to as SLB) (Bubeck 1992), gray leaf spot (GLS) (Bubeck et al. 1993; Clements et al. 2000; Maroof et al. 1996), common rust (Brown et al. 2001; Kerns et al. 1999), common smut (Luebberstedt et al. 1998), maize streak virus (Pernet et al. 1999), and aflatoxin accumulation in ears (Paul et al. 2003). BAC-based *in silico* mapping anchored two RGAs, sharing conserved protein kinase (PK) domain with *Pto* in tomatoes and *Pbs1* in Arabidopsis, to bins 8.05 and 8.06 (Xiao et al. 2006; Xiao et al. 2007). Several DRHs, including five members of the S-adenosyl methionine synthetase family involved in amino acid metabolism and an oxalate oxidase-like protein gene associated with hypersensitive

responses, were mapped to the same region using genetic and *in silico* analysis (Wang et al. 2007a).

Bin 8.05-8.06 is also a locus accounting for a significant proportion of NLB resistance in maize germplasm. NLB, caused by *Setosphaeria turcica* (anamorph *Exserohilum turcicum*, syn. *Helminthosporium turcicum*), is a foliar disease of maize that causes periodic epidemics associated with significant yield losses (Perkins and Pedersen 1987; Raymundo and Hooker 1981; Ullstrup and Miles 1957) in most maize-growing regions of the world. In diverse biparental populations, a number of NLB QTL (Schechert et al. 1999; Welz et al. 1999a; Welz et al. 1999b) as well as two major gene loci, *Ht2* (Yin et al. 2003; Zaitlin et al. 1992) and *Htn1* (Simcox and Bennetzen 1993), have been mapped to bin 8.05-8.06. Evaluation of a large multiparental mapping population (known as the nested association mapping population) (McMullen et al. 2009; Yu et al. 2008), consisting of 5000 recombinant inbred lines developed from 25 diverse maize lines, identified two largest effect NLB QTL in the same region (Poland et al. 2009b). In response to a recurrent selection program for NLB resistance (Ceballos et al. 1991), significant changes in allele frequencies provided evidence of selection acting at several loci in bin 8.05-8.06 (Wisser et al. 2008). To date, in the maize – *S. turcica* pathosystem, clustering of major genes and QTL has only been observed at bin 8.05-8.06 (Wisser et al. 2006).

As part of a larger attempt to capture diverse alleles at important resistance loci, we selected the maize genotype DK888 as a source of potentially useful alleles. This genotype has been shown to harbor alleles for resistance to diverse diseases (Kraja et al. 2000) and derived lines have been produced. In the present study, we aimed to fine-map and characterize DK888 allele(s) in bin 8.05-8.06 and to determine their disease- and race-specificity. Identification of the genes underlying the QTL region will be an important basis for detailed mechanistic studies.

The “heterogeneous inbred family” (HIF) approach was utilized to rapidly generate near-isogenic lines (NILs) carrying contrasting alleles at bin 8.05-8.06 (Tuinstra et al. 1997). This approach involves extraction of NILs from nearly-fixed lines, such as lines that have been produced by selfing segregating materials for several generations. Being isogenic at most of the genome but contrasting for specific QTL of interest, the HIF-derived NILs have been used to validate the position and effect of QTL (Borevitz and Chory 2004; Kobayashi et al. 2006; Loudet et al. 2005; Pumphrey et al. 2007). Compared to the NILs generated by successive backcrossing, NILs derived from HIFs can be put to use in a shorter period of time (particularly if nearly-isogenic lines are available, as they were in this case), and can possibly provide recombinant genetic backgrounds in which the QTL effects are well expressed (Tuinstra et al. 1997).

This study was undertaken to genetically dissect a complex genetic region associated with qualitative and quantitative resistance to NLB and a range of other diseases in maize. In order to identify, validate and characterize QTL, we isolated bin 8.05-8.06 of DK888, a maize line carrying favorable alleles for multiple disease resistance (Chapter 2; Kraja et al. 2000) in NILs using HIF-based approach. We will hereafter identify this QTL with bin 8.06, as it was initially located to a region spanning the distal end of bin 8.05 to the distal end of bin 8.06 (mostly in bin 8.06), and was ultimately fine-mapped to bin 8.06. NILs differing for the specific region were investigated to gain insights into a series of questions, including the disease- and race-specificity of the QTL, the QTL in relation to the known co-localized major gene loci, and the gene action at the QTL. To further unravel the complex genetic architecture and defense mechanisms, high-resolution mapping was conducted using break-point analysis. Our study has laid the foundation for positional cloning of a *S. turcica* race-specific resistance gene(s) underlying bin 8.06 of maize. The markers

closely linked to the major NLB QTL can also be used for practical resistance breeding.

MATERIALS AND METHODS

1. Plant materials

The initial plant materials for QTL identification were 17 F₆ heterogeneous inbred families (HIFs) from the cross of S11 x DK888, which were provided by The USDA Germplasm Enhancement of Maize (GEM) Project (Balint-Kurti et al. 2006; Goodman 2005; Lee and Hardin 1997). DK888 is a single-cross hybrid developed by Thailand Charoen Seeds Group in collaboration with US Dekalb Seeds. It was released in Thailand in 1991, and dominated the local Thailand hybrid maize seed market with 50% of the average in 1990s (Ekasingh et al. 2001). DK888 is a maize genotype carrying favorable alleles for resistance to NLB, southern leaf blight, gray leaf spot, northern leaf spot and common rust (Kraja et al. 2000). It also exhibited high levels of resistance to common smut and Stewart's wilt in our repeated field trials (details in Chapter 2). The subsequently derived HIFs and NILs were generated by single-seed descent from selected lines in the families segregating for bin 8.06. In this study, "NILs" refers to sets of HIF-derived F₈, F₉, F₁₀ and F₁₁ lines that contrasted for bin 8.06 but were presumably isogenic at > 99.2% of the genome.







Two sets of isolines with and without the *Ht* major genes were obtained from Peter Balint-Kurti of the USDA-ARS unit at North Carolina State University (a total of six differential lines: Pa91, Pa91*Ht1*, Pa91*Ht2*, Pa91*Ht3*, B68, and B68*Htn1*). *Ht1*, *Ht2*, and *Htn1* were derived from maize lines GE440, NN14B and Peptilla, respectively, while *Ht3* was derived from *Tripsacum dactyloides* (M. Carson, pers. comm.). Several F₁ and F₂ populations were developed by crossing the differential lines with the F₉

NILs carrying DK888 or S11 alleles at the QTL region. The differential lines were also used to provide reference phenotypes of major gene resistance to *S. turcica*.

2. Disease evaluations

Northern leaf blight. Resistance to NLB was evaluated with *S. turcica* race 1 (isolate EtNY001) in a greenhouse at Cornell University, and at Cornell's Robert Musgrave Research Farm in Aurora, NY from 2006-2009. The isolate EtNY001, originally collected from an infected leaf collected in Freeville NY in 1983, is compatible on Pa91*Ht1*, and incompatible on Pa91*Ht2*, Pa91*Ht3*, and B68*Htn1* (Table 3.1) under the standard greenhouse conditions established for NLB assays (Leonard et al. 1989). Another four *S. turcica* isolates representing different races, including Et10a (race 0), Et1001A (race 1), Et86A (race 23), and Et28A (race23N), were obtained from P. Balint-Kurti, and used exclusively for the race-specificity tests in the greenhouse. In the greenhouse, plants at the five to six-leaf stage were inoculated with 0.5 ml of spore suspension (4×10^3 conidia per ml in 0.02% Tween 20) in the whorl, and kept in a mist chamber at > 85% RH overnight. In the field, plants at the same stage were inoculated with spore suspension along with colonized sorghum grains (1/4 teaspoon, ~1.25 ml) in the whorl. The use of both liquid and solid inoculum was intended to ensure the viability of inoculum under dry weather conditions. *S. turcica* was cultured on lactose – casein hydrolysate agar (LCA) for two to three weeks, under a 12 hr/ 12 hr normal light-dark cycle at room temperature. Liquid inoculum was prepared by dislodging the conidia from the plates with sterilized ddH₂O, filtering the suspension through four layers of cheesecloth, and adjusting the concentration with the aid of a haemocytometer. Solid inoculum was prepared by inoculating autoclaved sorghum grains (900 ml of grains soaked overnight in 600 ml of dH₂O prior to autoclaving) with 1/5-1/3 of the suspension of spores and mycelium dislodged from a

Table 3.1 Race identification of EtNY001. A New York isolate of *Setosphaeria turcica* (EtNY001), was evaluated for race type on two sets of maize isolines with and without *Ht* major genes. Experiments were conducted under the standard greenhouse conditions established for race identifications (Leonard et al. 1989). Representatives of race 0 (Et10a), race 1 (Et1001A), race 23 (Et86A), and race 23N (Et28A) of *S. turcica* were included as positive controls. Pa91, Pa91*Ht1*, Pa91*Ht2* and Pa91*Ht3* were tested in November 2006 (five plants, one replication) and September 2008 (two replications, seven to eight plants per replication). B68 and B68*Htm1* were tested in August 2006 (two replications, three to eight plants per replication) and September 2008 (two replications, seven to eight plants per replication). The 2006 data from Pa91*Ht1* were not included for analysis, as the plants were weak and unusually susceptible for all races. Et86A (race 23) was only tested in 2008. Photographs were taken 20 days after inoculation. Trait values are least squares means calculated from the linear least squares model with “maize genotype”, “environment”, and “replication nested within environment” as independent variables. (IP: incubation period, measured as dpi, days post inoculation; PrimDLA: primary DLA, scored at 17 dpi as % of diseased leaf area on inoculated leaves). For each isolate, significant differences (represented as different letters) among the maize lines were determined by Tukey-Kramer HSD (honestly significant difference) tests at $P < 0.05$. Race-specific interactions were examined by comparing disease symptoms on the backcross lines carrying major genes, against the recurrent inbred lines (Pa91*Ht1*, Pa91*Ht2* and Pa91*Ht3* vs. Pa91; B68*Htm1* vs. B68). The incompatible responses, indicated as *, were based on the significant trait differences and the distinct resistant-type lesions. As a result, EtNY001 was identified as race 1, according to its compatibility with *Ht1*, and incompatibility with *Ht2* and *Ht3* and *Htm1*.

Maize differential lines						
	Pa91	Pa91Ht1	Pa91Ht2	Pa91Ht3	B68	B68Hm1
EtNY001 (identified as race 1)						
						
IP (dpi)	10.7 CD	10.8 CD	12.8 B*	12.2 BC*	10.5 D	14.6 A*
PrimDLA (%)	51.0 A	51.7 A	28.3 B*	31.7 B*	57.9 A	19.6 B*
Et10a (Race 0)						
IP (dpi)	11.4 C	14.8 B*	14.1 B*	14.2 B*	11.7 C	20.6 A*
PrimDLA (%)	53.5 A	23.8 B*	23.3 B*	26.4 B*	57.5 A	0.4 C*
Et1001A (Race 1)						
IP (dpi)	13.3 C	13.0 C	15.8 B*	14.9 B*	10.1 D	18.4 A*
PrimDLA (%)	28.3 B	21.7 BC	9.0 DE*	12.6 CD*	47.6 A	0.8 E*
Et86A (Race 23)						
IP (dpi)	9.9 B	12.2 A*	9.7 B	9.8 B	10.0 B	12.3 A*
PrimDLA (%)	60.4 B	27.0 A*	53.8 B	50.8 B	57.9 B	30.0 A*
Et28A (Race 23N)						
IP (dpi)	9.8 A	12.0 B*	9.6 A	9.9 A	9.6 A	10.1 A
PrimDLA (%)	70.0 A	49.6 B*	68.8 A	64.2 A	70.8 A	61.7 A

well-colonized LCA plate. The inoculated sorghum grains were shaken every day until use to prevent caking and to accelerate fungal colonization. Incubation period (IP) was rated as the number of days post inoculation (dpi) when observing the appearance of first wilted lesion on a plant. IP was checked every day until 25 dpi. The 50% IP was recorded on a row basis when > 50% of the plants in a row started showing the lesions. Primary DLA (PrimDLA) was rated as the percentage of infected leaf area of the inoculated leaves for individual plants at two to three weeks after inoculation. DLA was rated as the percentage of infected leaf area of the entire plant, disregarding decayed bottom leaves for individual plants or on a row basis for fixed lines. DLA was recorded two to three weeks after the onset of secondary infection.

Southern leaf blight. Resistance to SLB was evaluated with *Cochliobolus heterostrophus* race O in the greenhouse in September 2007, and in Clayton, North Carolina in 2008. In the greenhouse trial, plants at the five to six-leaf stage were inoculated with the isolate C5 (ATCC 48332) obtained from G. Turgeon at Cornell University. Inoculum was cultured on complete medium with xylose (CMX) under continuous fluorescent light for 7-10 days, and spore suspension was prepared as described for NLB. About 0.5 ml of spore suspension (5×10^4 conidia per ml, 0.02% Tween 20) was evenly sprayed on the first fully expanded leaf with an airbrush (Badger® Model 150) at 20 psi. After inoculation, the plants were kept in a mist chamber at > 85% RH overnight. Lesion length was measured at 4 dpi from 20 randomly chosen lesions on each plant. Primary DLA was rated at 6 dpi as the percentage of infected leaf area of the inoculated leaf. In the field trial, plants at the four to six-leaf stage were inoculated as previously described (Carson 1998; Carson et al. 2004). Disease severity was rated based on a 1 to 9 scale corresponding to the diseased leaf area on primarily the ear leaf. Disease was evaluated for three times at 10 to 12-day intervals from around two weeks after anthesis. The disease severity

scores were used to calculate area under the disease progress curve (AUDPC) by
$$\text{AUDPC}_{\text{Severity}} = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})(t_{i+1} - t_i)}{2},$$
 where y_i = disease severity at time i , $t_{i+1} - t_i$ = day interval between two ratings, n = number of ratings.

Gray leaf spot. Resistance to naturally occurring GLS (caused by *Cercospora zea-maydis* and/or *Cercospora zeina*) was evaluated in Blacksburg, Virginia in 2008. The non-tillage field was located in a valley with regular morning mists and heavy dews, conditions that favor GLS development. Disease severity was scored based on a 1 to 10 scale with 0.25 increments, according to the disease progress on the ear leaf (Saghai Maroff et al. 1993). The evaluation was conducted four times at a 7 to 8-day intervals from about two weeks after anthesis. The $\text{AUDPC}_{\text{Severity}}$ was calculated as described above.

Anthraxnose leaf blight. Resistance to anthracnose leaf blight (ALB) was evaluated in the greenhouse in September 2007 and September 2008, with *Colletotrichum graminicola* (teleomorph: *Glomerella graminicola*) isolate Cg151 (obtained from G. Bergstrom of Cornell University). Inoculum was cultured on oatmeal agar for two weeks under continuous fluorescent light at room temperature (Muimba-Kankolongo and Bergstrom 1990). Each plant at the five to six-leaf stage was inoculated in the whorl with 0.5 ml of spore suspension (2×10^4 conidia per ml, 0.02% Tween 20, prepared as described above), then kept in a mist chamber at > 85% RH overnight. Individual plants were rated for IP, latent period (LP) and PrimDLA. LP was rated as the number of dpi when observing the first appearance of black acervuli on the lesions. The ratings of IP and PrimDLA were as described for NLB.

Anthraxnose stalk rot. Resistance to anthracnose stalk rot (ASR) was evaluated with *C. graminicola* isolate Cg151 in the greenhouse in December 2007, and in Aurora NY in 2008. For each plant at tasseling stage (Keller and Bergstrom 1988), the first internode above the brace root was punctured with an ice pick, a 1 ml pipette tip

was inserted, and the plant was inoculated with 1 ml of spore suspension (10^6 conidia per ml, 0.02% Tween 20, prepared as described for ALB) through the tip. At four weeks post inoculation, the stalk of each individual plant was split longitudinally, and the percentages of discolored area were rated for eight (the trial in 2007) or six (2008) consecutive internodes (Keller and Bergstrom 1988). Data from all the scored internodes were summed for analysis.

Common rust. Resistance to rust was evaluated in the greenhouse in September 2007, and in Aurora, New York in 2008, with urediniospores of *Puccinia sorghi* collected from naturally infected leaves at Aurora NY in 2007. In the greenhouse trial, about 200-300 mg of stock urediniospores (preserved at -80°C) were suspended in 100 ml of Sortrol oil (Chevron Phillips Chemical Company, Phillips, TX, USA) (Webb et al. 2002). About 1 ml of suspension was evenly applied on the first two fully expanded leaves of each plant with a spray gun (Preval, Yonkers, NY, USA). Plants were kept in a mist chamber at $> 85\%$ RH overnight. Individual plants were observed daily and rated for first pustule appearance, which is the number of dpi when the first pustule on a plant is observed. Pustules on the inoculated leaves were counted at 10 dpi. PrimDLA was rated at 14 dpi as described above. For the field trial, inoculum was increased on three to four-leaf stage seedlings of susceptible sweet corn inoculated in the greenhouse. The urediniospores were collected by agitating infected leaves with matured rust pustules in distilled water, and filtering the spores through four layers of cheesecloth. Field plants at six to eight-leaf stage were inoculated with 1 ml of spore suspension (2×10^5 urediniospores per ml, 0.02% Tween 20) in the whorl (Pataky and Campana 2007). Disease severity was rated on a row basis using a 0-10 scale with 0.5 increments, corresponding to the percentage of infected leaf area of the entire plant (0 = no disease, 1 = 10%, ..., 10 = 100%). The $\text{AUDPC}_{\text{Severity}}$ was calculated as described above, from three severity scores evaluated at 9-day intervals from four weeks after

inoculation.

Common smut. Resistance to smut was evaluated in the greenhouse in November 2007, and in Aurora, NY in 2008, with six compatible strains of *Ustilago maydis* (UmNY001, UmNY002, UmNY003, UmNY004, UmNY008 and UmNY009) which were isolated from naturally infected smut galls collected in Aurora, NY in 2007 [isolation procedure: (Thakur et al. 1989b); compatibility test: (Puhalla 1968)]. The first ear of each plant was shoot-bagged, and injected with 2 ml of sporidial suspension (10^6 sporidia per ml in 0.02% Tween 20) through the silk channel, when the silk had emerged 1-5 cm. Inoculum was prepared by culturing the isolates separately in potato-dextrose broth (PDB) on a shaker at 100 rpm at room temperature for 1 day, adjusting the sporidial concentrations with sterilized ddH₂O, and mixing equal amounts of compatible strains right before inoculation (du Toit and Pataky 1999). In the greenhouse trial, the volume (length x width x height) and weight of ear galls were measured. In the field trial, the incidence and severity of ear galls and naturally occurring stalk galls were rated at four to five weeks post anthesis. Severity scores were evaluated for individual plants on a 0-10 scale, corresponding to the number and size of galls, and the disease severity of the entire plant.

Stewart's wilt. Resistance to Stewart's wilt was evaluated with *Pantoea stewartii* (syn. *Erwinia stewartii*) strain PsNY003 (obtained from H. Dillard of Cornell University) in Aurora, NY in 2008. Plants at the five to six-leaf stage were inoculated following a modified pinprick method (Blanco et al. 1977; Chang et al. 1977). Whorl leaves of each plant were pierced twice with a specialized inoculator pre-dipped in bacterial suspension [10^7 colony forming units per ml in sterilized 0.1 M NaCl solution, prepared as described by Suparyono and Pataky (1989)]. Multiple-pin inoculators was made with 30 T-pins (1.5 inch long), pieces of 5.5 cm x 6.5 cm sponge, and cork board (3/8 inch thick) fastened on two arms of a tong with rubber

bands. PrimDLA (as described for NLB) was rated on a row basis at two and three weeks after inoculation.

3. Genotyping assays

DNA extraction. Plant genomic DNA was extracted following a modified mini-prep CTAB method (Doyle and Doyle 1987; Qiu et al. 2006). The high-throughput extraction was conducted using 96-well plates (Corning® Costar 96 Well Polypropylene Cluster Tubes). For each sample, about 0.1 g of leaf tissue was frozen and ground with a stainless steel ball (5/32 inch diameter, OPS Diagnostics, NJ, USA), at 450 strokes per min for 50-120 sec using Genogrinder 2000 (SPEX CertiPrep Inc., Metuchen, NJ, USA). Pulverized sample was suspended in 500 µl of CTAB extraction buffer [2 % (w/v) hexadecyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.2% (v/v) of 2-mercaptoethanol; 2-mercaptoethanol was added prior to use], and incubated at 65 °C for 30-50 min. The CTAB suspension was mixed thoroughly with 400 µl of chlorophorm/isoamyl alcohol (24:1, v/v) for 3 min, then centrifuged at 5200 rpm for 15 min. The supernatant was transferred to a new tube, mixed with 300 µl of isopropanol, and incubated at -20 °C overnight. DNA was precipitated by centrifuging the sample at 5200 rpm at 4 °C for 12 min, and recovered by repeatedly discarding the supernatant and rinsing with 70% then 100% ethanol. The air-dried DNA pellet was dissolved in 100-150 µl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Simple sequence repeat (SSR) markers. SSR primers were chosen from the Maize Genetics and Genomics Database (MaizeGDB) (<http://www.maizegdb.org/>). To integrate the fluorescent dye in the PCR product, the specific primer pair and a fluorescently-labeled universal primer were used in a single-reaction nested PCR

(Schuelke 2000). Each PCR reaction was performed as described by Wisser *et al.* (2008) in a total volume of 13 μ l, with the same thermal cycling parameters as described by Schuelke (2000). The resulting amplicons labeled with different dyes were multiplexed (up to four PCR reactions were combined) and analyzed with the Applied BioSystems 3730xl DNA Analyzer at Biotechnology Resource Center at Cornell University. Each sample consisted of 0.7 μ l PCR product per primer pair, 0.05-0.1 μ l GeneScan-500 LIZ size standard, and 9 μ l formamide (Applied Biosystems, Foster City, CA, USA). The sizes of amplicons were scored using GeneMapper v. 3.0 (Applied Biosystems).

Single nucleotide polymorphism (SNP) and cleaved amplified polymorphic site (CAPS) markers. The B73 genomic sequences were used as a reference map for identifying polymorphisms between DK888 and S11. Various genes across the QTL region were chosen as the templates for marker design. Gene sequences were obtained from the database of the Maize Genome Sequencing Project (the MaizeSequence database, www.maizesequence.org), and the specific primers for each gene were designed using Primer 3 (Rozen and Skaletsky 2000). Each PCR reaction was performed in a total volume of 16 μ l, containing final concentrations of 1x PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% (v/v) Triton X-100], 1.5 mM MgCl₂, 1 μ M forward-specific primer, 1 μ M reverse-specific primer, 1-3 units Taq polymerase, and 20-50 ng template DNA. The thermal cycling parameters for different sets of primers can be found in Table 3.2. PCR products amplified from DK888 and S11 homozygotes were purified with exonuclease I and shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA, USA), and sequenced at Biotechnology Resource Center at Cornell University. The DNA sequencing was performed using BigDye Terminator and AmpliTaq-FS DNA Polymerase, and analyzed on the Applied BioSystems 3730xl DNA Analyzer (Applied Biosystems). The sequencing results

Table 3.2 Single nucleotide polymorphism (SNP) markers and cleaved amplified polymorphic site (CAPS) markers around *qNLB8.06*.

Marker ^a	Map position ^b			PCR amplification			Cleavage of PCR product	
	Physical map position (Mb)	Genetic map position (cM)	Gene template	Primer (5' to 3') (F: forward, R: reverse)	Thermal cycling parameters ^c	Size of PCR product (bp) ^d	Specific restriction endo-nuclease	Restriction site (allele, bp) ^e
<u><i>ctg358-03</i></u>	141.85	1.09	ZmEvi088378	F: TCTTGTTCCATTGCCAGAGC R: TGCATGAAGATGTGCAGACG	C1, C2	597	Bsu36I	S11: 180
<u><i>ctg358-07</i></u>	142.41	2.34	AC187246.5_FG017	F: TGGTGTTCGGTTCTACGCTTC R: TCCTGGTCTTTGAGGGCATC	C1, C2, C3	534	BsiHKA I	S11: 281
<u><i>ctg358-08</i></u>	142.78	3.26	AC202181.2_FG005	F: AAGTGTCTCCTTTACCTTCTGG R: GCCCCTTTTTGTGTGTAGGC	C1, C2, C3	555	BspCNI	DK888: 67, 229 S11: 68
<u><i>ctg358-09</i></u>	142.88	3.93	AC202181.2_FG023	F: TGAGGTGGA AAAATCCGAACC R: CACAGGAAGAGAGCGTCATCA	C1, C2, C3	759		
<u><i>ctg358-13</i></u>	143.69	9.53	AC202388.3_FG026	F: GGAGCCGTGACTGAGAAAGAT R: GATGATTGGCAGGTTTGCTG	C1, C2, C3	582		
<u><i>ctg358-14</i></u>	143.88	9.78	AC215232.2_FG020	F: CCCTCCTCCGCTCTGTCTTT R: ACCGTCGCTGTGGCTCTATT	C1, C2, C3	502	StyI	DK888: 62, 135
<u><i>ctg358-16</i></u>	143.88	9.86	AC215232.2_FG021	F: CTACGCACCCTTCATCCACA R: CGAGCACAAAGAACCAGAGC	C1, C2	839	StyI	S11: 256
<u><i>ctg358-18</i></u>	143.88	9.86	AC215232.2_FG021	F: AACATCCAGGGCGAGTGTCT R: AGCATCGGTTAGGGTTTCCA	C1, C2	709	NdeI	DK888: 259
<u><i>ctg358-20</i></u>	143.92	10.20	AC215232.2_FG028	F: CAGCGTCCAACAACACATCC R: ACGACCTCCGACTCCTACCC	C1, C2, C3	663	DraIII	DK888: 494
<u><i>ctg358-05</i></u>	144.06	10.28	AC197148.2_FG023	F: GAGCATGCCTTCCGACAAAT R: TTGGTACAACGCTGGCAAAG	C1	550	AclI	DK888: 142
<u><i>ctg358-37</i></u>	144.07	10.28	AC197148.2_FG026	F: ACTCCTCTCGCTCCCAGACA R: TCGCCAGTGACCCAGTAAATC	C1	610		

Table 3.2 (Continued)

Marker ^a	Map position ^b			PCR amplification			Cleavage of PCR product	
	Physical map position (Mb)	Genetic map position (cM)	Gene template	Primer (5' to 3') (F: forward, R: reverse)	Thermal cycling parameters ^c	Size of PCR product (bp) ^d	Specific restriction endonuclease	Restriction site (allele, bp) ^e
<u>ctg358-44</u>	144.38	11.20	AC199378.1_FG002	F: CTAGCTCTACTTTCGTGCAGTGT AAGCCTAGGAG R: GTACACAACGTCCACCGTCTC TCTTGCCTTTAG	TD	854	BamHI	S11: 225
<u>ctg358-32</u>	144.56	11.33	AC199378.3_FG038	F: CAACCTCTCCTCCCTCCAGA R: CAGCGTTCCCTTCCAGTGA	C1, C2, C3	758	BanI	DK888: 520
<u>ctg358-33</u>	144.56	11.33	AC199378.3_FG038	F: GCTTGCCTGTTTCATCTGGT R: TCGCTCTTCCTTGACGCTTT	C1, C2	807		
<u>ctg358-01</u>	144.65	11.97	AC199186.2_FG004	F: CTCCCAGAGGTGTCGTGTTG R: TGGTCTGTTCAACGGTGTCC	C2	513	AccI	S11: 302
<u>ctg359-01</u>	146.05	13.41	AC195822.2_FG005	F: GACCTCTCCCTGACCCACAC R: TAGACTCGGGGAAAGGCAAA	C2	542		
<u>ctg359-02</u>	146.65	14.03	ZmEvi066814	F: AGATCGTGGGGATGGGTATG R: ATCAGCTGGCCTTTGCTCTC	C2	522	MnII	S11: 153
<u>ctg360-02</u>	148.15	20.34	ZmEvi050510	F: GTTCGGAGGATCTCGACAGG R: GAGAAGCAGTGGAGCCGAAT	C1	552	ApoI	S11: 129
<u>ctg360-04</u>	148.45	21.63	AC199708.2_FG028	F: CGAGAGGGTGGCTCCTACTG R: CAGAACATCATCGCCTCCAG	C2	526	BsrDI	S11: 111

^a The markers with and without underline are CAPS and SNP markers, respectively.

^b The map position is based on the B73 physical map and the S11 x DK888 genetic map.

^c Conventional PCR (annealing temperatures – C1: 56 °C, C2: 60 °C, C3: 62 °C) and touchdown PCR (TD) (Don et al. 1991) were employed for different sets of primers.

- C1, C2, or C3: 1 cycle of 95 °C for 4 min, 30 cycles of 95 °C for 1 min / 56, 60, or 62 °C for 1 min / 72 °C for 1 min, and 1 cycle of the final extension at 72 °C for 10 min.
- TD: 1 cycle of 95 °C for 4 min, 10 cycles of 95 °C for 1 min / 65-60 °C for 1 min (starting from 65 °C, 0.5 °C decrease per cycle) / 72 °C for 1 min, 25 cycles of 95 °C for 1 min / 60 °C for 1 min / 72 °C for 1 min, and 1 cycle of the final extension at 72 °C for 10 min.

^d The approximate size of PCR product was estimated from the gene sequences of B73 line.

^e The allele-specific restriction site(s) (bp) in the amplicon.

were then aligned and analyzed for SNPs, small indels (insertions/deletions), and restriction sites using BioLign version 2.0.9 (developed by T. Hall, <http://en.bio-soft.net/dna/BioLign.html>). CAPS markers were developed if restriction-site polymorphisms were detected. For CAPS markers, PCR products were completely digested with specific restriction endonucleases (New England Biolabs), and the resulting polymorphic fragments were revealed using standard agarose gel electrophoresis followed by ethidium bromide staining. The SNP and CAPS markers are listed in Table 3.2.

4. Genetic map

A genetic map of 11 SSR markers spanning the *qNLB8.06_{D K888}* region was constructed using genotypic data from segregating F₉ families. The map distances between SSR markers were estimated using MapDisto 1.7.0 (Lorieux 2007) based on Kosambi's mapping function (Kosambi 1944). The relative genetic distances between the 12 and 7 newly-developed SNP markers in the intervals of *umc2199 - umc2210* and *umc2210 - umc1287*, respectively, were calculated by the proportion of identified crossover events between SSR markers. Corresponding physical positions of the markers were obtained from the physical map of the inbred line B73, resulting from the MaizeSequence database.

5. QTL analysis

Single-marker analysis and interval mapping (Lander and Botstein 1989) were performed using Windows QTL Cartographer 2.5 (Wang et al. 2007b) to analyze QTL position in segregating heterogeneous inbred families. In interval mapping, QTL were scanned at a walk speed of 0.5 cM. The threshold values were based on the likelihood of odds ratio (LOD) scores from 1000 permutations of the original at a significance

level of $P = 0.01$ (Churchill and Doerge 1994). The LOD threshold used in the study was averaged from the threshold value calculated for each trait. For the marker locus closest to the QTL peak, the additive effect and the proportion of phenotypic variance explained by the QTL (R^2) were obtained using the Windows QTL Cartographer. The R^2 values for single marker analysis were from the analysis of variance (ANOVA) conducted in JMP 7.0 (SAS Institute Inc., Cary, NC, USA). The allele effect was designated as the mean difference between DK888 homozygotes and S11 homozygotes at a locus. The 95% confidence interval for the QTL was estimated according to the “1-LOD support interval”, which includes the QTL peak and its right and left loci with LOD scores dropping within 1 (Lander and Botstein 1989).

6. Experimental design and statistical analysis

HIFs for the identification and fine-mapping of the QTL. From 2006-2008, individual plants in each heterogeneous inbred family were genotyped for segregating markers, and phenotyped for resistance to NLB in a controlled greenhouse at Cornell University, or in Aurora, NY (Table 3.3). To control environmental variations, plants in a family were grown within a single block. Data were analyzed using Windows QTL Cartographer 2.5 as described in “QTL analysis”. Analysis of variance (ANOVA) was also carried out on an individual trait-marker basis using JMP 7.0. The phenotypic differences among different genotypes were determined by pairwise two-tailed Student’s t test at $P < 0.05$.

F₈ and F₉ NILs for the characterization of the QTL. Derived F₈ and F₉ NILs were evaluated for resistance to a range of important diseases, and to different races of *S. turcica*. A pair of F₈ NILs was grown in separate field plots or greenhouse blocks for different disease evaluations. In the field, plants were put in side-by-side rows with B73 and DK888 rows as controls (two replications, 10 seeds per line per replication in

Table 3.3 Summary of QTL analysis for *qNLB8.06*_{DK888} in segregating heterogeneous inbred families (HIFs) derived from S11 x DK888. Resistance to northern leaf blight (NLB) was evaluated in the greenhouse or field with disease components including: incubation period (IP), primary diseased leaf area on inoculated leaves, and/or diseased leaf area on entire plants. In single marker analysis, the marker closest to the QTL peak, and its corresponding likelihood of odds ratio (LOD), allele effect, and proportion of phenotypic variance explained by QTL (R^2), are reported. In interval mapping, the marker interval covering the 95% confidence interval for QTL position (1-LOD support interval) is reported. The LOD, allele effect and R^2 were from the marker closest to QTL peak.

Mapping population (numbers of HIFs)	Sample size	Phenotyped sample size	Environment	Trait	Single marker analysis					Marker interval	Interval mapping			
					Nearest marker	QTL Position (cM) ^a	LOD	Allele effect ^b	R^2 ^c		QTL interval (cM) ^a	LOD	Allele effect ^b	R^2
1 F_{6:7}	53	53	Aurora NY, 06	IP	umc1149	38.33	2.7	5.9 days	0.21	–	–	–	–	–
1 F_{7:8}	96	96	GH, Apr-Jun 07	IP	umc1287	22.40	15.0	6.8 days	0.62	–	–	–	–	–
				PrimDLA			9.5	-22.4 %	0.38	–	–	–	–	–
12 F_{8:9}	571	225 ^d	GH, Oct-Dec 07 ^e	IP	umc1287	22.40	77.6	2.6 days	0.32	umc2199 - umc1287 ^e	0 – 25.40 ^e	29.4	2.9 days	0.59
				PrimDLA			31.2	-11.7 %	0.14			7.9	-16.1 %	0.19
13 F_{9:10}	1191	745 ^d	GH, Apr-Jun 08	IP	ctg358-20	10.20	86.2	4.7 days	0.45	ctg358-18 – ctg358-44	9.86 – 11.20	97.6	5.7 days	0.47
14 F_{8:9}	1056	1056	Aurora NY, 08	IP	ctg358-20	10.20	77.8	4.0 days	0.35	ctg358-18 – ctg358-44	9.86 – 11.20	96.3	5.2 days	0.35
				DLA	ctg358-05 ctg358-37	10.28	172.6	-14.9 %	0.60			210.8	-18.6 %	0.60

^a The map position is based on the genetic map constructed using F_{8:9} families derived from S11 x DK888. The genetic map and the likelihood of the presence of QTL are shown in Figure 5.

^b The allele effect is the difference between DK888 homozygotes and S11 homozygotes at the marker closest to the QTL peak.

^c The R^2 values for single marker analysis were calculated from the analysis of variance (ANOVA) performed in JMP 7.0. All the other data were retrieved from the output of Windows QTL Cartographer 2.5.

^d In space-limited greenhouse, recombinant individuals for target region were selected for phenotyping.

^e The resistance was not as effective in this environmental condition. The QTL interval was estimated conservatively (not based on the 1-LOD support interval).

each year); in the greenhouse, each evaluation consisted of two replications in two blocks, with six to eight plants per NIL per replication. The race specificity tests were conducted in the greenhouse, using a pair of F₈ NILs in September 2007, and a set of six F₉ NILs (four NILs with *qNLB8.06*_{DK888}, and two NILs with *qNLB8.06*_{S11}) in September 2008. Each treatment (per race by NIL) consisted of two replications placed in two separate blocks, with six to eight plants per replication. Lines were randomized within blocks. In the trial in 2008, the maize differential lines Pa91, Pa91*Ht1*, Pa91*Ht2*, Pa91*Ht3*, B68, and B68*Htn1* were also included as control (two replications, seven to eight plants per replication). Data were analyzed using JMP 7.0 by fitting linear least squares models with “*S. turcica* race by the allele at *qNLB8.06*”, “environment” and “replication nested within environment” as independent variables. For the data from a single environment, “*S. turcica* race by the allele at *qNLB8.06*” and “replicate” were used as the variables. Differences among the least squares means of the “race by allele” were determined by Tukey-Kramer HSD (honestly significant difference) test at $P < 0.05$.

F₁₀ and F₁₁ NILs for high-resolution mapping of the QTL. A total of 13 F₁₀ and 85 F₁₁ NILs were evaluated at Aurora NY in 2008 (for IP and DLA) and 2009 (for IP only), respectively. The NILs were put in rows (10 seeds per row) with two replications per year. In 2008, the 13 F₁₀ NILs were randomized within each replication, with DK888 and B73 rows as control. In 2009, the 85 F₁₁ NILs originated from 11 F₉ families were grown in 11 randomized blocks, according to their parental families (NILs from the same F₉ line were randomized in one block). Two extra control rows, originating from the corresponding F₉ lines, were grown on one side of each block. The resistant and susceptible control rows were two F₁₀ NILs homozygous for DK888 and S11 alleles for the entire QTL region (*umc2199* - *umc1287*). In addition to the IP and DLA ratings, the NILs were classified as “resistant” or

“susceptible” based on the comparisons with the control lines in the same block. Using JMP 7.0, data from 2008 and 2009 were analyzed separately by fitting a mixed model with each “marker” as a fixed factor, and “replications” and “blocks within replications” as random effects. The analyses were performed on an individual marker – trait basis. Significance levels of marker – QTL associations were represented by the negative logarithm P -values ($-\text{Log } P$) converted from the resulting F -statistics.

F₁ and F₂ populations for allelic analysis. In 2009, the allelic relationships between *qNLB8.06_{DK888}* and *Ht2*, and between *qNLB8.06_{DK888}* and *Htn1*, were evaluated in the greenhouse and in the field at Aurora, NY, respectively. The F₁ and F₂ progenies derived from different pairs of Pa91, Pa91*Ht2*, and B68*Htn1* crossed with the NIL carrying DK888 or S11 allele at *qNLB8.06*, were individually phenotyped for IP, PrimDLA (scored at 18 days after inoculation; only in the greenhouse trial), and lesion types. Two F₁₀ NILs contrasting for *qNLB8.06*, Pa91, Pa91*Ht2*, B68, and B68*Htn1* were used as control. In the greenhouse, the evaluation consisted of two replications, four blocks per replication, with five to six plants per control lines, five to six plants per F₁ population and 10-12 plants per F₂ population randomized in each block. Data from F₁ progenies and control lines were analyzed using JMP 7.0 by fitting a linear least squares model with “genotype”, “replication” and “block nested within replication” as independent variables. Differences among the least squares means of genotypes were determined by Tukey-Kramer HSD (honestly significant difference) test at $P < 0.05$. In the field, plants were put in rows with 10 seeds per row (average germination rate was 38%). Plants in each population (10 rows per F₁ population, and 24 rows per F₂ population) were grown in one block, with control rows on the side. Data were analyzed as described above, with only “genotype” and “block” as the variables.

7. Identification of candidate genes

Putative genes in the B73 genomic sequences have been predicted by the Maize Genome Sequencing Project using the Gramene pipeline (Liang et al. 2009) (data available at the MaizeSequence database, www.maizesequence.org). The evidence-based gene prediction was conducted by aligning the sequences of known proteins, full-length cDNAs, and expressed sequence tags (ESTs) from maize as well as cross-species libraries to the bacterial artificial chromosome (BAC) contigs of B73. We surveyed existing predicted genes spanning the fine-mapped QTL interval. The potential identities of the predicted coding sequences were subsequently determined by performing BLAST (basic local alignment search tool) searches at the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

RESULTS

1. Identification of an incompletely dominant NLB QTL (*qNLB8.06_{DK888}*) by HIF analysis

Following the HIF methodology described by Tuinstra *et al.* (1997), the study's first step was to detect residual heterozygosity at potential disease QTL regions in the HIFs derived from S11 x DK888. Forty-six individuals of 17 F₆ families (one to four individuals per family) were analyzed with 17 markers covering 12 bins. The marker targeting bin 8.06 was *umc1149*. An individual heterozygous for *umc1149* and another marker at bin 5.06 (*umc2216*) was identified, and was used to generate the genetic materials for subsequent QTL analysis.

In 2006, a F₇ family consisting of 53 individuals was evaluated for resistance to NLB (Table 3.3). The F₇ progeny were segregating for *umc1149* and *umc2216*, but

isogenic at ~98.4 % of the genome. Variation in disease response co-segregated with *umc1149* (DK888 allele for resistance; allele effect in IP = 5.9 dpi, LOD = 2.7, $R^2 = 0.21$), not *umc2216*, indicating the existence of a candidate NLB QTL at bin 8.06. In 2007, the finding was further validated in a F₈ family (96 individuals) segregating for *umc1149* but fixed at *umc2216*. Consistently, the QTL contributed strong effects on reducing disease. As much as 62% and 38% of phenotypic variation in IP and PrimDLA, respectively, were explained by the QTL (Table 3.3).

To more precisely localize the identified NLB QTL, an additional 15 SSR markers across bins 8.05-8.06 were used to estimate the start and end points of heterozygous loci in the HIFs. Assuming that each end of the QTL segment lies halfway between the last marker for the introgression and the first marker outside it, the QTL was determined to reside in the interval of 386.8-453.7 cM on the IBM 2008 neighbors map, and between 136.2-156.0 Mb on the B73 physical map. This is a region spanning bins 8.05 and 8.06, but located mostly in bin 8.06. Among the nine markers analyzed in the F₈ family (*umc1287*, *umc1828*, *umc2356*, *umc1149*, *bnlg240*, *umc1997*, *umc1728*, *umc2361*, *umc2395*), the QTL was closest to *umc1287*.

The identified QTL locating mostly in bin 8.06, designated as *qNLB8.06*, showed incompletely dominant resistance (Fig. 3.1). It was observed that the level of resistance in DK888 homozygotes was much greater than in the heterozygotes or in the S11 homozygotes. The magnitudes and significance levels of the differences among the three genetic classes (PrimDLA: DK888/DK888 – S11/S11 = -22.0 %, $P < 0.0001$; DK888/DK888 – heterozygotes = -14.1 %, $P < 0.0001$; heterozygotes – S11/S11 = -7.9 %, $P = 0.005$) suggested that the resistance performance in heterozygotes is relatively more similar to S11 homozygotes. The same type of gene action was consistently seen in the subsequent mapping populations.

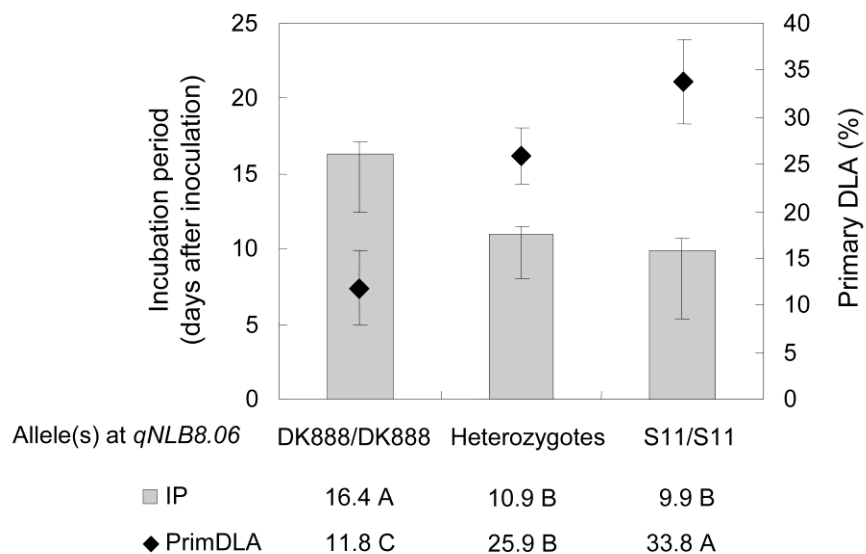


Figure 3.1 Gene action at *qNLB8.06*. DK888 homozygotes showed greater resistance than the heterozygotes and S11 homozygotes, suggesting that resistance conferred by the DK888 allele(s) is incompletely dominant. The phenotypic differences among the three genetic classes were tested in an F₈ family by ANOVA, followed by pairwise Student's t test at $P < 0.05$.

2. *qNLB8.06_{DK888}* is not effective for multiple disease resistance

To understand the resistance spectrum of *qNLB8.06_{DK888}*, a pair of F₈ NILs was characterized for resistance to GLS, SLB, ALB, ASR, common rust, common smut and Stewart's wilt (Table 3.4). The NILs derived from a single F₇ line were contrasting for the QTL region but isogenic at ~99.2% of the genome, according to the theoretical level of heterozygosity in F₈ progeny. Based on the trials conducted in 2007-2008 in the field and/or controlled greenhouse, no significant differences were found between the NIL pairs for response to any of the seven diseases. The result suggested that although DK888 harbors multiple disease resistance, the resistance conferred by *qNLB8.06_{DK888}* is NLB-specific.

3. *qNLB8.06_{DK888}* conditions race-specific resistance

Race-specific responses in IP, PrimDLA and lesion type were observed for the NILs (F₈ and F₉) carrying DK888 allele(s) at *qNLB8.06*. As shown in Fig. 3.2, race 0 and race 1 were avirulent to *qNLB8.06_{DK888}*, while race 23 and race 23N were highly virulent to it. Typical resistance symptoms caused by the incompatible interactions between *qNLB8.06_{DK888}* and race 0/race 1 were characterized by prolonged IP, decreased DLA and resistant-type lesions. The resistant-type lesions were slightly chlorotic and more restricted, in contrast to the susceptible-type lesions which extended greatly after the first appearance. The chlorosis, likely induced by the hypersensitive response surrounding the infection sites, was more distinct in early stages of lesion development. Once the pathogen grew out from the localized primary infected region, the resistant or susceptible reactions were differentiable by size rather than the type of mature lesion.

The defense mechanism conferred by *qNLB8.06_{DK888}* was ineffective when inoculated with race 23 and race 23N. The observed race specificity suggested that the

Table 3.4 Resistance spectrum of *qNLB8.06*_{DK888}. The NILs carrying DK888 or S11 alleles at bin 8.06 were evaluated for resistance to a range of important diseases in maize, including gray leaf spot (GLS), southern leaf blight (SLB), anthracnose leaf blight (ALB), anthracnose stalk rot (ASR), common rust, common smut, and Stewart's wilt. Different disease components were applied in the field and greenhouse. No significant contrasts were observed between the NIL pairs, indicating *qNLB8.06*_{DK888} is not effective for any of the diseases.

Disease	Parameter	Unit	Allele(s) at <i>qNLB8.06</i> in the NIL		<i>P</i> -value ^b
			DK888 ^a	S11 ^a	
GLS	AUDPC _{Severity} ^f	area unit	55.7 ± 2.1	57.1 ± 2.1	0.47
SLB	Lesion length ^g	mm	1.2 ± 0.05	1.2 ± 0.05	0.72
	Primary diseased leaf area ^g	%	29.5 ± 1.2	30.0 ± 1.2	0.58
	AUDPC _{Severity} ^f	area unit	27.8 ± 3.7	23.7 ± 2.6	0.33
ALB	Incubation period ^g	days after inoculation	7.9 ± 0.2	7.9 ± 0.2	0.80
	Latent period ^g	days after inoculation	9.8 ± 0.3	9.8 ± 0.3	0.99
	Primary diseased leaf area ^g	%	44.0 ± 6.7	46.7 ± 6.7	0.57
ASR	Discolored internode area ^{gf}	Total % of internode	102.5 ± 10.1	105.8 ± 9.4	0.63
Common rust	First postule appearance ^g	days after inoculation	7.5 ± 0	7.5 ± 0	0.99
	Number of pustules ^g	# pustules	163.9 ± 51.7	149.5 ± 46.8	0.71
	Primary diseased leaf area ^g	%	14.4 ± 3.1	15.0 ± 2.7	0.79
	AUDPC _{Severity} ^f	area unit	46.1 ± 2.2	46.1 ± 2.2	0.99
Common smut	Volume of ear gall ^g	cm ³	273.8 ± 129.3	167.5 ± 123.3	0.26
	Weight of ear gall ^g	grams	127.4 ± 57.6	78.9 ± 54.9	0.25
	Incidence of ear gall ^f	%	29.0 ± 10.0	23.0 ± 10.0	0.49
	Severity of ear gall ^f	scale	1.8 ± 0.6	1.0 ± 0.6	0.19
	Incidence of stalk gall ^f	%	0.0 ± 0.0	0.0 ± 0.0	0.99
	Severity of stalk gall ^f	scale	0.0 ± 0.0	0.0 ± 0.0	0.99
Stewart's wilt	Primary diseased leaf area ^f	%	72.5 ± 4.9	72.5 ± 4.9	0.99

^a Trait values are 95% confidence intervals of least squares means, calculated from the linear least squares model with “allele(s) at *qNLB8.06*”, “environment” and “replication nested within environment” as independent variables. For the data from a single environment, “allele(s) at *qNLB8.06*” and “replication” were used as the variables.

^b Two-tailed Student's *t* test was conducted on the difference between least squares means of the NIL pairs.

^f Disease parameters evaluated in the field.

^g Disease parameters evaluated in the greenhouse.

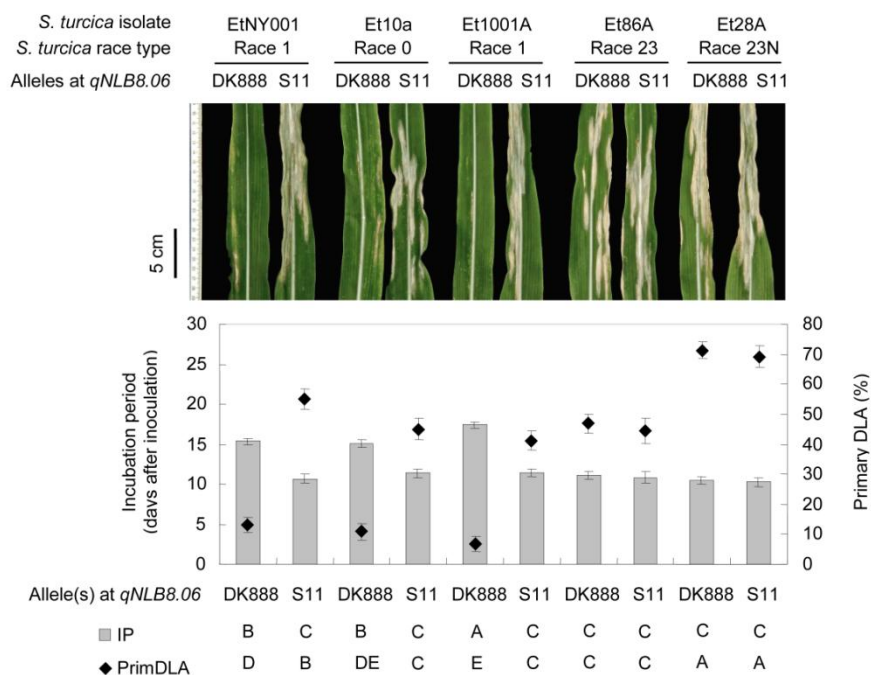


Figure 3.2 Race specificity of *qNLB8.06*_{DK888}. The F₈ and F₉ NILs carrying DK888 or S11 alleles at bin 8.06 were evaluated for resistance to different races of *S. turcica*. Photographs were taken 20 days after inoculation. Trait values (IP: bars; primary DLA: dots, scored at 17 days after inoculation) are least squares means calculated from the linear least squares model with “*S. turcica* race by *qNLB8.06* allele”, “environment”, and “replication nested within environment” as independent variables. The error bars represent the 95% confidence interval of the least squares means. Determined by Tukey-Kramer HSD (honestly significant difference) test at $P < 0.05$, significant differences for IP and primary DLA were indicated as different letters below the graph. The result provided evidence that *qNLB8.06*_{DK888} conditions resistance to race 0 and race 1, but not race 23 and race 23N of *S. turcica*.

QTL could coincide with the major genes *Ht2*, *Ht3*, and/or *Htn1*. While *Ht3* locus has not been mapped, *Ht2* and *Htn1* loci have been mapped to bin 8.05-8.06, which suggests that *qNLB8.06_{DK888}* may encompass *Ht2* and/or *Htn1*, or some novel modulator(s) conditioning the expression of *Ht2* and/or *Htn1*. *Ht2* is a creditable candidate for *qNLB8.06_{DK888}* based on the compatibility of race 23. The relationship between *qNLB8.06_{DK888}* and *Htn1* is ambiguous, as the compatibility of race 23N may have been caused by *Ht2* and *Htn1*, or *Ht2* alone. However, there is no naturally-occurring race N isolate available for further resolving the question. It is also worth noting that the resistance reactions of *qNLB8.06_{DK888}* did not fully resemble those on the maize differential lines Pa91*Ht2* or B68*Htn1*. As illustrated by the control trials (Table 3.1), the lesions on Pa91*Ht2* were more chlorotic associated with accumulated reddish pigmentation, and the lesions on B68*Htn1* were of the susceptible type, consistent with previously reported lesion types of *Ht2* and *Htn1* (Welz and Geiger 2000). In contrast to *qNLB8.06_{DK888}*, *Ht2* and *Htn1* were effective in delaying lesion formation by 2-3 days and 2-9 days, respectively.

Significant differences in IP and PrimDLA were detected among the same lines inoculated with either different compatible or incompatible isolates. For instance, all the lines with S11 alleles at *qNLB8.06* were compatible with *S. turcica*, but isolate Et28A caused more severe symptoms than the other isolates did. The variation in disease severity approximately reflected the growth rates of individual isolates on LCA plates (Et28A > EtNY001 \approx Et86A > Et10a > Et1001A, data not shown). Thus, the aggressiveness of the isolates *in planta* was apparently correlated with aggressiveness *in vitro*.

4. Allelism with known major genes at *qNLB8.06*

qNLB8.06 in relation to Ht2. To understand the allelism and interactions between *qNLB8.06_{DK888}* and *Ht2*, the F₁ and F₂ progenies of *qNLB8.06_{DK888}* x

Ht2_{NN14B}, *qNLB8.06_{DK888}* x *Ht2_{Pa91}*, *qNLB8.06_{S11}* x *Ht2_{NN14B}*, and *qNLB8.06_{S11}* x *Ht2_{Pa91}* were evaluated in the greenhouse (Fig. 3.3). *Ht2_{NN14B}* represented the resistance allele (from the donor line NN14B) at the *Ht2* locus in the isolate Pa91*Ht2*, and *Ht2_{Pa91}* represented the susceptible allele in the recurrent line Pa91. As expected, all the F₁ and F₂ individuals of *qNLB8.06_{S11}* (S) x *Ht2_{Pa91}* (S) were susceptible. In contrast, no susceptible plants were found in either F₁ or F₂ individuals of *qNLB8.06_{DK888}* (R) x *Ht2_{NN14B}* (R) (Fig. 3.3b). Distinct chlorotic-necrotic lesions were observed in almost all the plants derived from *qNLB8.06_{DK888}* (R) x *Ht2_{NN14B}* (R). No susceptible individuals were observed, though three out of 35 F₁ individuals and six out of 72 F₂ individuals showed an intermediate phenotype on lower leaves, which is possibly caused by incomplete expression of resistance under low light intensity (Reuveni et al. 1993; Thakur et al. 1989a). Complementation of the DK888 and NN14B alleles in resistance phenotypes suggests that *qNLB8.06_{DK888}* is likely to be identical, allelic, or closely linked to the *Ht2* locus.

Significantly different levels of NLB resistance were observed in the four F₁ progenies with the same hybrid background: *qNLB8.06_{DK888}* x *Ht2_{NN14B}* > *qNLB8.06_{DK888}* x *Ht2_{Pa91}* > *qNLB8.06_{S11}* x *Ht2_{NN14B}* > *qNLB8.06_{S11}* x *Ht2_{Pa91}* (Fig. 3.3e). Although they showed some levels of resistance, the F₁ progenies of neither *qNLB8.06_{DK888}* x *Ht2_{Pa91}* (Fig. 3.3c) nor *qNLB8.06_{S11}* x *Ht2_{NN14B}* (Fig. 3.3d) showed typical resistant chlorotic-necrotic lesions, indicating incomplete dominance of the *qNLB8.06_{DK888}* and *Ht2_{NN14B}* alleles. The quantitative difference between the F₁ progenies of *qNLB8.06_{DK888}* x *Ht2_{Pa91}* and *qNLB8.06_{S11}* x *Ht2_{NN14B}* also suggested differential allelic effects, though the effectiveness of individual alleles could not be determined.

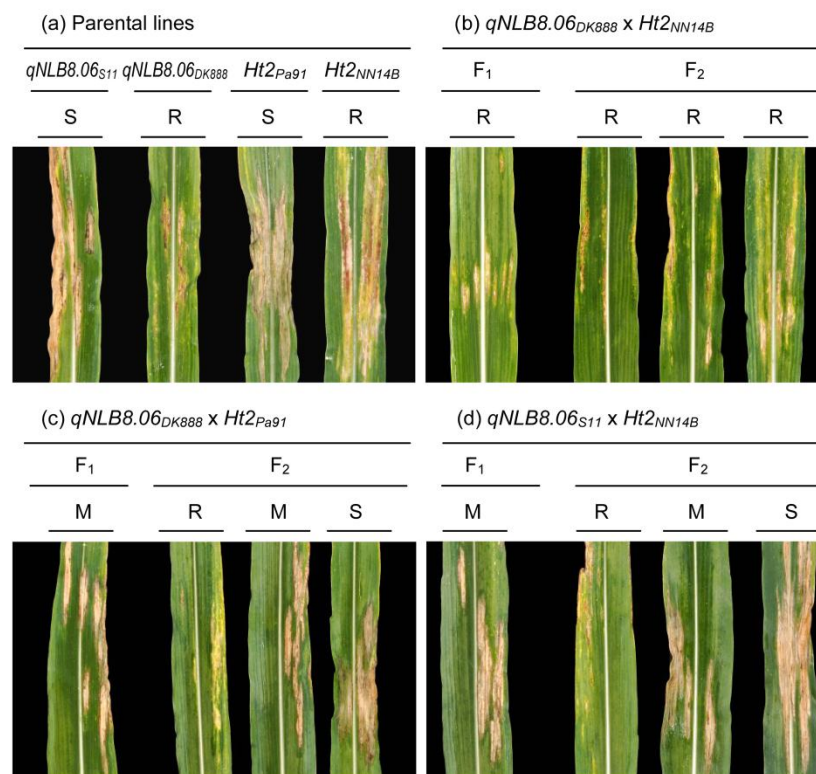
As expected, marked segregation of resistant, intermediate and susceptible phenotypes was observed in the F₂ populations from the crosses of *qNLB8.06_{DK888}* (R)

x *Ht2*_{Pa91} (S) and *qNLB8.06*_{S11} (S) x *Ht2*_{NN14B} (R) (Fig. 3.3c and 3.3d). The intermediate phenotypes in heterozygotes complicated the classification of resistant and susceptible plants. We decided not to pursue Mendelian segregation ratio test on the F₂ populations, as the analysis would provide meaningful results only if based on complete dominant or recessive genes with high penetrance. Nevertheless, careful observation and ratings were still conducted, from which the incomplete dominance of the *qNLB8.06*_{DK888} and *Ht2*_{NN14B} alleles, and the likely differential allelic effects were confirmed.

Induced accumulation of reddish pigmentation surrounding chlorotic-necrotic lesions was associated with the *Ht2*_{NN14B} allele and/or Pa91 genetic background. Extensive reddish pigmentation was consistently observed on diseased leaves of Pa91*Ht2* (Fig. 3.3a). In contrast, the pigmentation was never seen on the NILs carrying *qNLB8.06*_{DK888} or *qNLB8.06*_{S11}, or their derived lines. All the F₁ and F₂ progenies used in this allelism test, however, showed different degrees of accumulated pigmentation. Relative to the F₂ progeny derived from *qNLB8.06*_{DK888} x *Ht2*_{Pa91}, more individuals with higher degrees of reddish pigmentation were seen in the F₂ populations of *qNLB8.06*_{DK888} x *Ht2*_{NN14B} and *qNLB8.06*_{S11} x *Ht2*_{NN14B}. Variation in the pigmentation was also seen in the F₂ population of *qNLB8.06*_{DK888} x *Ht2*_{NN14B}. The variation implies the involvement of the gene(s) controlling the biosynthesis of anthocyanins. These results, however, did not clearly differentiate between an influence of the *qNLB8.06*(*Ht2*) locus or the genetic background.

qNLB8.06 in relation to Htn1. To understand the allelism and interactions between *qNLB8.06*_{DK888} and *Htn1*, the F₁ and F₂ progenies of *qNLB8.06*_{DK888} x *Htn1*_{Peptilla} and *qNLB8.06*_{S11} x *Htn1*_{Peptilla} were evaluated in the field (Fig. 3.4). *qNLB8.06*_{DK888} x *Htn1*_{B68} and *qNLB8.06*_{S11} x *Htn1*_{B68} were not included due to

Figure 3.3 Analysis of allelism between *qNLB8.06_{DK888}* and *Ht2*. (a) Crosses were made between the near-isogenic lines (NILs) contrasting for bin 8.06 (alleles designated *qNLB8.06_{S11}* and *qNLB8.06_{DK888}*), Pa91 (alleles designated *Ht2_{Pa91}*), and Pa91*Ht2* (alleles designated *Ht2_{NN14B}*). Plants carrying homozygous *qNLB8.06_{DK888}* showed chlorotic-necrotic resistance lesions, and plants carrying homozygous *Ht2_{NN14B}* showed chlorotic-necrotic resistance lesions with accumulated reddish pigmentation. The F₁ and F₂ progenies of *qNLB8.06_{DK888}* × *Ht2_{NN14B}* (b), *qNLB8.06_{DK888}* × *Ht2_{Pa91}* (c), *qNLB8.06_{S11}* × *Ht2_{NN14B}* (d), and *qNLB8.06_{S11}* × *Ht2_{Pa91}* (not shown) were evaluated for resistance to race 1 of *S. turcica* (EtNY001) in the greenhouse. (b) Complementation between the *qNLB8.06_{DK888}* and *Ht2_{NN14B}* alleles in resistance phenotypes was observed. (c) (d) Intermediate phenotype (less susceptible-type lesions) was observed in all the F₁ individuals and a considerable proportion of the F₂ individuals, suggesting that the resistance conditioned by either *qNLB8.06_{DK888}* or *Ht2_{NN14B}* was incompletely dominant. (e) Significant differences in incubation period (bars) and primary diseased leaf area (dots, scored at 18 days after inoculation) were observed among the four F₁ progenies. The F₁ individuals were comparable, as they differed at bin 8.06 and *Ht2* but isogenic for the rest of the genome. Trait values are least squares means calculated from the linear least squares model with “genotype”, “replication” and “block nested within replication” as independent variables. Differences were determined by Tukey-Kramer HSD (honestly significant difference) test at $P < 0.05$, and indicated as different letters below the graph. The result confirmed the incomplete dominance of *qNLB8.06_{DK888}* and *Ht2_{NN14B}*, and implicated the potential existence of different alleles at bin 8.06. Photographs were taken on the 6th leaves at 19 days after inoculation. Disease phenotypes are denoted as R: resistant, M: intermediate, and S: susceptible.



(e) Comparison of the F₁ progenies from different crosses

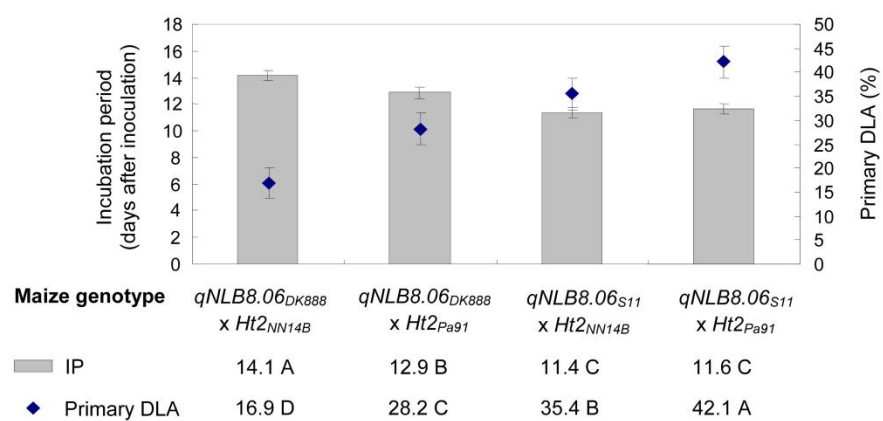
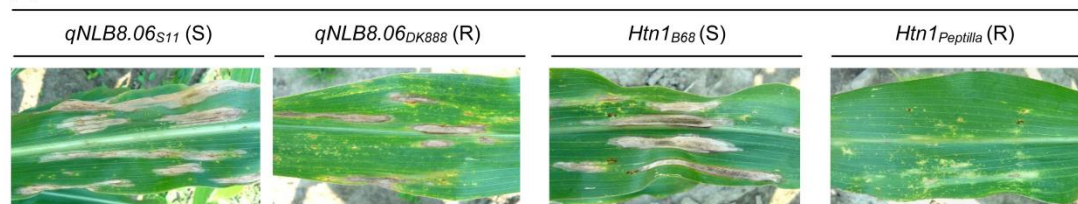
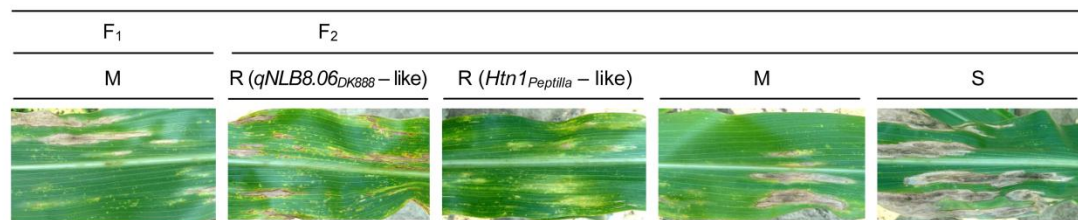


Figure 3.4 Analysis of allelism between *qNLB8.06_{DK888}* and *Htn1*. (a) Crosses were made between the near-isogenic lines (NILs) contrasting for bin 8.06 (alleles designated *qNLB8.06_{S11}* and *qNLB8.06_{DK888}*), and B68*Htn1* (alleles designated *Htn1_{Peptilla}*). The line B68 (alleles designated *Htn1_{B68}*) was used as control. Plants carrying homozygous *qNLB8.06_{DK888}* showed chlorotic-necrotic resistance lesions, and plants carrying homozygous *Htn1_{Peptilla}* showed extraordinarily delayed formation of lesions (until 25 days after inoculation, only a few lesions were observed on B68*Htn1*). The F₁ and F₂ progenies of *qNLB8.06_{DK888}* x *Htn1_{Peptilla}* (b) and *qNLB8.06_{S11}* x *Htn1_{Peptilla}* (c) were evaluated for resistance to race 1 of *S. turcica* (EtNY001) in the field. (b) *qNLB8.06_{DK888}* and *Htn1_{Peptilla}* appeared to be non-allelic, based on the segregation of plants exhibiting chlorotic-necrotic lesions, delayed lesion formation, intermediate phenotypes, and susceptible lesions in their F₂ progeny. (c) The resistance conditioned by *Htn1_{Peptilla}* was incompletely dominant. Photographs were taken on the 7th leaves at 25 days after inoculation. Disease phenotypes are denoted as R: resistant, M: intermediate, and S: susceptible.

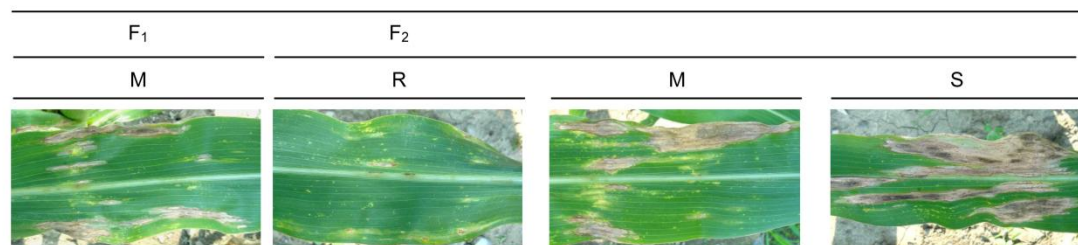
(a) Parental lines



(b) *qNLB8.06_{DK888}* x *Htn1_{Peptilla}*



(c) *qNLB8.06_{S11}* x *Htn1_{Peptilla}*



unavailability of seed. *Htn1_{Peptilla}* represented the resistance allele (from the donor line Peptilla) at the *Htn1* locus in the isoline B68*Htn1*, and *Htn1_{B68}* represented the susceptible allele in the recurrent line B68. Similar to *qNLB8.06_{DK888}* and *Ht2_{NN14B}*, *Htn1_{Peptilla}* was much less effective in the heterozygous than homozygous state. Homozygous *Htn1_{Peptilla}* in B68*Htn1* (average IP > 25 dpi) increased IP by more than 13 days relative to B68 (average IP = 13.8 dpi). Heterozygous *Htn1_{Peptilla}* in the F₁ progeny of *qNLB8.06_{S11}* x *Htn1_{Peptilla}* (average IP = 15.1 dpi, Fig. 3.4c), however, only increased IP by 1.3 days ($P = 0.050$) and 2.3 days ($P = 0.0002$) relative to B68 line and the NIL carrying *qNLB8.06_{S11}* (average IP = 12.8 dpi), respectively. The incomplete dominance of *Htn1* has been described (Raymundo et al. 1981). It was also observed that when *Htn1_{Peptilla}* and *qNLB8.06_{DK888}* were both heterozygous, the plants displayed an intermediate resistant phenotype that was characterized by slightly chlorotic-necrotic lesions (Fig. 3.4b) and moderately increased IP [average IP = 18.6 dpi, significantly different from and in between of homozygous *Htn1_{Peptilla}* (average IP > 25 dpi) and homozygous *qNLB8.06_{DK888}* (average IP = 15.9 dpi)]. The intermediate phenotype conforms to previously reported phenotype resulting when heterozygous *Htn1* interacts with heterozygous *Ht2* (Simcox and Bennetzen 1993). This implies some functional similarity of *qNLB8.06_{DK888}* and *Ht2*.

Unlike the complementation of *qNLB8.06_{DK888}* and *Ht2_{NN14B}*, resistance phenotypes of homozygous *qNLB8.06_{DK888}* (chlorotic-necrotic), homozygous *Htn1_{Peptilla}* (extremely prolonged IP), and heterozygous *qNLB8.06_{DK888}* in combination with heterozygous *Htn1_{Peptilla}* (intermediate) segregated in the F₂ progeny of *qNLB8.06_{DK888}* x *Htn1_{Peptilla}* (Fig. 3.4b). The Mendelian segregation ratio test was not employed because of the ambiguity in phenotypic classification. Nonetheless, it was clearly observed that four out of 82 F₂ individuals showed a susceptible phenotype (shorter IP with extended long lesions), which is presumably associated

with recombination events between *qNLB8.06* and *Htn1*. The result indicates that *qNLB8.06* and *Htn1* are non-allelic. In view of the results of the allelism analysis, the locus designation was modified to *qNLB8.06(Ht2)*.

5. Fine-mapping of *qNLB8.06(Ht2)*

Breakpoint analysis was conducted to refine *qNLB8.06(Ht2)_{DK888}*. Around 2800 individuals (from 26 F₉ families and 13 F₁₀ families) segregating for bin 8.06 were used for QTL analysis. Disease evaluations were carried out in three environmental conditions: Oct-Dec in the greenhouse, Apr-Jun in the greenhouse, and May-Aug in the field (Table 3.3). In the space-limited greenhouse, plants were initially all genotyped for flanking markers of the target QTL interval. Subsequently, only the identified recombinant individuals were kept for disease evaluations. The mapping results from single-marker analysis and interval mapping are summarized in Table 3.3, and displayed in the QTL likelihood map in Fig. 3.5.

In a population consisting of 571 F₉ individuals, *qNLB8.06(Ht2)_{DK888}* was found to be likely located between *umc2199* - *umc1287*. The LOD scores at this interval were > 10 for IP and > 5 for PrimDLA, whereas the LOD values dropped to less than 3 from around 27 cM to 58 cM (between *umc1287* - *umc1828*). In this particular greenhouse trial conducted during the winter, the resistance of *qNLB8.06(Ht2)_{DK888}* was not as effective as in other environments. The QTL contributed to the delay of lesion formation by 2-3 days, which is ~2 days shorter than the average delaying effect observed in the same greenhouse during the summer and in the field. The resistant-type lesions were not as distinct either. The shorter IP difference and indistinguishable resistance symptoms may have caused some phenotyping errors. As a result, although the QTL interval can be estimated, the QTL peak should be interpreted with caution.

A high resolution map was constructed with 19 newly developed SNP and CAPS

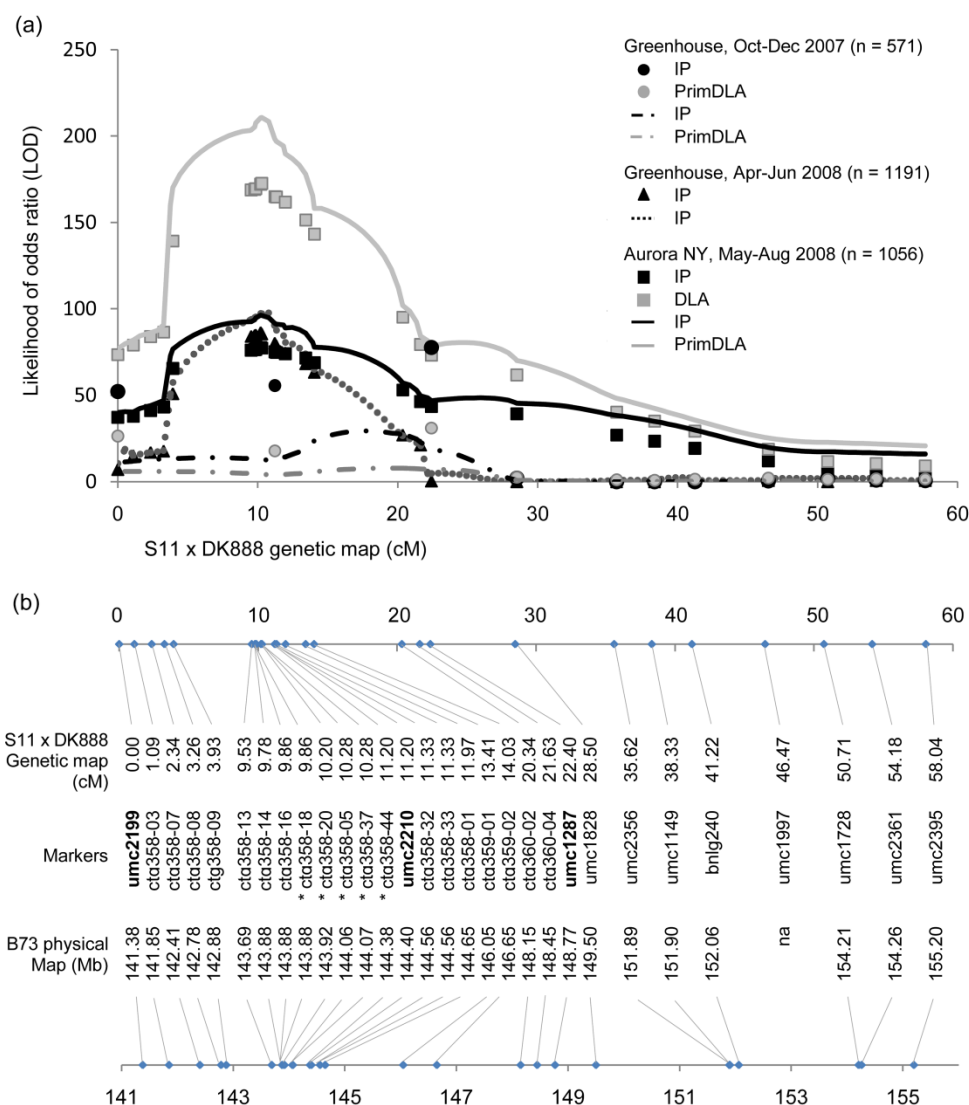


Figure 3.5 Likelihood map of $qNLB8.06(Ht2)_{DK888}$. (a) The likelihood that the loci in bin 8.06 are associated with NLB resistance was analyzed by single marker analysis (circle, triangle, and square dots) and interval mapping (solid and dash lines). The LOD lines and dots for incubation period (IP) are shown in black, and the LOD lines and dots for primary DLA (PrimDLA) and DLA are shown in gray. The average LOD threshold for all traits, based on 1000 permutations at $P = 0.01$, is 3.3 (not shown in the figure). The resistance of $qNLB8.06(Ht2)_{DK888}$ was not as effective in the greenhouse trial conducted in Oct-Dec 2007. The most likely QTL position, based on the data obtained from the evaluations in the greenhouse in Apr-Jun 2008 and in the field in May-Aug 2008, was located between *ctg358-18* to *ctg358-44*. This interval corresponds to 9.86-11.20 cM on the S11 x DK888 genetic map, and 143.88-144.38 Mb on B73 physical map. (b) The genetic and physical positions of the markers are shown.

markers around *umc2199* - *umc1287* (Fig. 3.5). Initially, two and five SNP/CAPS markers between *umc2199* - *umc2210* and *umc2210* - *umc1287*, respectively, were designed to cover the QTL region at low density. By testing the co-segregation of markers and traits, the interval of *ctg358-07* - *ctg358-01* was found to be the most significantly associated with resistance. An additional 12 SNP/CAPS markers were then developed to saturate this region. Marker segregation data showed that the order of the SSR, SNP and CAPS markers used in the study agree with their physical positions in the genome sequence of B73.

In order to increase efficiency, individuals in mapping populations were selectively genotyped for SNP/CAPS markers, based on their genotypes at the flanking markers of the target interval. Considering the incompletely dominant gene action of *qNLB8.06(Ht2)_{DK888}*, the genotyping strategy aimed to capture the homozygous DK888 segment(s), which provided informative resistance phenotype for QTL analysis. Therefore, all the recombinant individuals that were homozygous for the DK888 allele at either one of the flanking markers were genotyped for intermediate SNP/CAPS markers. The recombinants that were heterozygous at one flanking marker, and homozygous for S11 allele at the other flanking marker were not tested, since the probability of finding a homozygous DK888 segment in between was very low. In this case, the interval marker loci were all assigned as “S11/ –” for QTL analysis (the minus sign “–” represents an unknown allele). Individuals that were homozygous for identical alleles at the two flanking markers were assumed homozygous for the entire interval.

In a population consisting of 1191 F₁₀ individuals and a population consisting of 1056 F₉ individuals, *qNLB8.06(Ht2)_{DK888}* was delimited to a region of ~1.34 cM (~0.5 Mb) between *ctg358-18* - *ctg358-44* (Table 3.3 and Fig. 3.5). In the two experimental environments, the resistance of *qNLB8.06(Ht2)_{DK888}* was well expressed, allowing

accurate linkage analysis on the basis of distinct phenotypes. Averaged from the effects estimated from single marker analysis and interval mapping, the DK888 allele increased IP by ~5 days and decreased DLA by ~17%. About 35-47 % and 60% of the variance in IP and DLA, respectively, were explained by *qNLB8.06(Ht2)*_{DK888}. Significant evidence of QTL (LOD > 3.3, the average threshold for all traits) was consistently found between *umc2199* - *umc1287* (0-22.4 cM). In this interval, QTL peaks were detected at approximately the same map position (~10.2 cM) for IP and DLA (highest LOD scores: ~97 for IP, and ~210 for DLA). If adopting the 1-LOD drop method, the most likely QTL position can be predicted to a tight region between *ctg358-18* - *ctg358-44* (Fig. 3.5; 9.86-11.20 cM on the S11 x DK888 genetic map, and 143.88-144.38 Mb on B73 physical map).

To further confirm the location of *qNLB8.06(Ht2)*_{DK888}, a total of 13 F₁₀ and 85 F₁₁ NILs were evaluated for IP, DLA (only in the 2008 trial) and lesion types at Aurora NY in 2008 and 2009, respectively. The NILs were derived from selected lines covering different breakpoints around *umc2199* - *umc1287*. The result of single marker-trait analysis and the genotypic compositions of nine representative fixed NILs are shown in Fig. 3.6. (Evaluations conducted in 2008 and 2009 led to the same results. Since F₁₁ NILs captured more recombination events in more homogeneous backgrounds, the data from the 2009 trial was shown to represent the overall result.) Markers *ctg358-20*, *ctg358-05*, and *ctg358-37* were found to be the most significantly associated with disease traits (-Log of *P* values > 200, Fig. 3.6). Among the three markers, *ctg358-20* is likely to reside outside of the QTL region, based on the “resistant” phenotype of NIL7 (S11/S11 at *ctg358-20*, DK888/DK888 at *ctg358-05* and *ctg358-37*). Evidence of the QTL tightly linked to *ctg358-05* and *ctg358-37* was also found in the rows of NIL9 (Fig. 3.6; heterozygous at *ctg358-05* and *ctg358-37*), where individual plants segregated for resistance. The numbers of resistant:

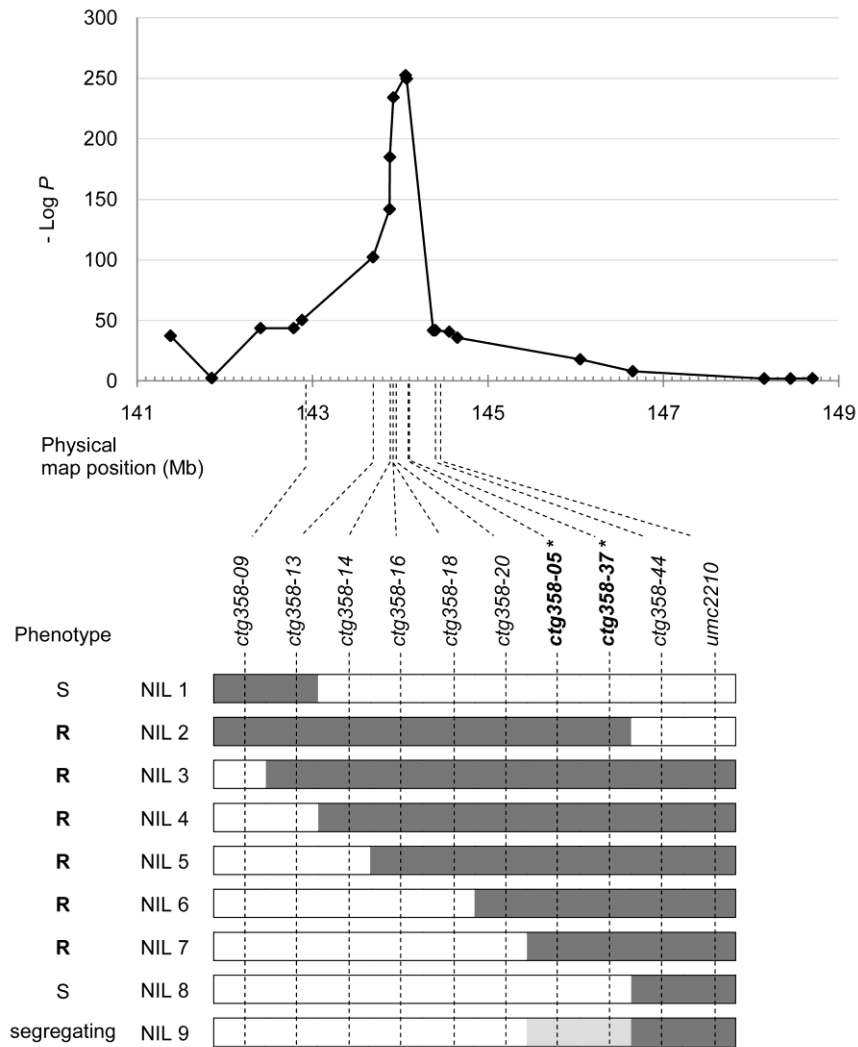


Figure 3.6 Validation of $qNLB8.06(Ht2)_{DK888}$ position. F_{10} and F_{11} near-isogenic lines (NILs) capturing various recombination events at bin 8.06 were evaluated for resistance to northern leaf blight (NLB). The likelihood of each locus being associated with incubation period is represented by negative logarithm P -values ($-\text{Log } P$) derived from a mixed model analysis. Genotypic compositions and disease phenotypes (R: resistant, S: susceptible, segregating: R and S plants segregating in a row) of nine representative NILs are shown. The solid bars and open bars represent the loci homozygous for DK888 alleles and S11 alleles, respectively. The gray bar represents heterozygous loci. $qNLB8.06(Ht2)_{DK888}$ was delimited to a map interval between *ctg358-20* and *ctg358-44* (10.20-11.20 cM on the S11 x DK888 genetic map, and 143.92-144.38 Mb on B73 physical map).

intermediate/susceptible plants were 4:21, which does not deviate from the expected 1:3 segregation ratio ($X^2 = 1.2$, $P = 0.3$) of a single incompletely dominant gene. *qNLB8.06(Ht2)_{DK888}* was thus validated to locate between (but not overlapping with) *ctg358-20* and *ctg358-44* in bin 8.06 (10.20-11.20 cM on the S11 x DK888 genetic map, and 143.92-144.38 Mb on B73 physical map). There is some ambiguity regarding the precise boundary between bins 8.05 and 8.06. The region 143.92-144.38 Mb was located to bin 8.06 in MaizeGDB, while it was located to a gap between bins 8.05-8.06 in the MaizeSequence database.

6. Candidate genes underlying *qNLB8.06_{DK888}*

On the basis of the annotation of the Maize Genome Sequencing Project (as of August 2009), the genomic region between *ctg358-20* and *ctg358-44*, spanning 0.46 Mb, harbors a large number of transposable elements (TEs) and 12 putative genes (GRMZM2G135202, GRMZM2G164612, GRMZM2G164640, GRMZM2G091973, GRMZM2G092018, GRMZM2G119720, GRMZM2G018260, GRMZM2G122912, GRMZM2G006188, GRMZM2G042017, GRMZM2G077187 and GRMZM2G065538). The abundance of TEs has been generally observed in the entire maize genome (Wei et al. 2007). Of the 12 non-TE genes, eight genes encode putative proteins with similarities to known protein domains or motifs in the InterPro databases. Putative genes that can be associated with previously reported R-genes or defense-related genes include two protein kinase-like genes (GRMZM2G135202 and GRMZM2G164612 with conserved domains IPR017441, IPR002290, IPR001245, IPR017442, IPR011009, IPR008271) and one serine-threonine specific protein phosphatase-like gene (GRMZM2G119720 with conserved domain IPR006186). The two protein kinase-like genes are closely linked (2632 bp apart) and highly homologous to each other (78% genomic sequence identity; 97% putative transcript

identity; putative proteins different for 1 out of 290 amino acid residues).

DISCUSSION

1. Production of near-isogenic lines (NILs) for a complex resistance locus using heterogeneous inbred families (HIFs)

NILs carrying contrasting alleles at maize bin 8.06 were successfully generated, and the region was characterized and dissected using HIF analysis. The HIF-based QTL approach was conducted as part of a larger effort to capture diverse alleles at the loci associated with complex types of disease resistance. To increase the probability of finding alleles conditioning broad-spectrum resistance, maize lines possessing multiple disease resistance were used as donors. In the present study, the broadly resistant maize line DK888 was used as a source of alleles. Considering the importance of bins 8.05-8.06 in NLB resistance (two major genes and many co-localized QTL have been mapped to the region), the effect of DK888 allele(s) at bin 8.06 was first tested for response to NLB.

We detected, validated and localized an NLB QTL at bin 8.06 (designated *qNLB8.06*) using initially one single SSR marker and subsequently 15 additional markers. The F₇ and F₈ families in which *qNLB8.06*_{DK888} was identified were segregating for putatively less than 1.6 % of the genome. In these HIFs, *qNLB8.06*_{DK888} appears to be a major QTL explaining a large proportion (14-62 %) of phenotypic variations in NLB resistance. *qNLB8.06*_{DK888} consistently conferred resistance in juvenile and adult plants across greenhouse and field environments. Relative to S11 allele(s), DK888 allele(s) at bin 8.06 was effective for delaying lesion formation by about 2.6-6.8 days, and reducing diseased leaf area by about 12-22 % of the primarily inoculated leaves and about 15% of the entire plant. Overall, HIF

analysis proved to be an efficient way to extract targeted QTL from the nearly fixed recombinant inbred lines (Tuinstra et al. 1997). Genetic stocks derived during the procedure were readily applicable for subsequent work of characterizing and fine-mapping QTL. Clear expression of the disease phenotypes in the NILs indicated that the QTL was transferred to an appropriate genetic background for QTL examination.

2. *qNLB8.06* conditions race-specific resistance to NLB

The hypothesis that DK888 allele(s) at bin 8.06 conditions disease- and race-nonspecific resistance was tested. The DK888 allele(s) at bin 8.06 conferred resistance only to NLB (among the several diseases tested). The resistance was also characterized by its specificity to race 0 and race 1, but not to race 23 and race 23N of *S. turcica*. The compatibility with race 23 and race 23N led to the question of whether *qNLB8.06_{DK888}* is the same or different from the known major genes *Ht2* and *Htn1*. We found that *qNLB8.06_{DK888}* is likely to be identical, allelic, or very closely linked (and functionally related) to *Ht2_{NNI4B}*, on the basis of their overlapping map locations, their similarities in race-specificity and resistance phenotypes, and their complementation for resistance in the F₁ and F₂ test progenies. *qNLB8.06_{DK888}* and *Htn1* appear to be linked and functionally dissimilar genes, according to the intermediate resistant phenotype in their F₁ progeny, and the segregation of F₂ individuals showing chlorotic-necrotic lesion type (typical *Ht2* phenotype), intermediate lesion type, or delayed formation of lesions (typical *Htn1* phenotype). These observations conformed to previously reported non-allelism of *Ht2* and *Htn1* (Simcox and Bennetzen 1993). *Htn1* was mapped to ~10 cM distal to *Ht2* in the F₂ progeny of W22*Htn1* x A619*Ht2*. In our group, concurrent work of fine-mapping *Htn1* using a population consisting of ~2600 F₂ individuals derived from B68 x B68*Htn1* is underway (J. Kolkman, pers. comm.). The map distance between

qNLB8.06(Ht2) and *Htn1* will be further clarified.

3. *qNLB8.06(Ht2)* shows incomplete dominance

Available evidence on gene action at *Ht2* and *Htn1* from previous studies is ambiguous. For *Ht2*, both complete dominance (Yin et al. 2003; Zaitlin et al. 1992) and incomplete dominance (Ceballos and Gracen 1989; Hooker 1977) have been observed in different genetic materials. Reduced resistance in the heterozygotes (incomplete dominance) and the vulnerability of resistance to genetic backgrounds have also been reported for *Htn1* (Raymundo et al. 1981). The effects of *Ht2* and *Htn1* have been found to be highly sensitive to environmental conditions in others' experiments (Reuveni et al. 1993; Thakur et al. 1989a) and our repeated greenhouse and field trials (data not shown). In the present study, both *DK888* and *NN14B* alleles at *qNLB8.06(Ht2)* conditioned incomplete dominance and race-specific resistance to *S. turcica* (*NN14B* is the resistance donor line used to derive Pa91*Ht2* isoline). High levels of resistance and the distinct chlorotic-necrotic lesions were only seen on the plants containing two copies of resistance alleles (*DK888/DK888*, *NN14B/DK888* or *DK888/NN14B*) at the locus. One copy of the resistance allele along with one copy of a susceptible allele resulted in differential intermediate degrees of disease and susceptible type lesions. The resistance performance of *qNLB8.06(Ht2)* in the heterozygous state is thus more quantitative than qualitative.

Incomplete dominance has been widely observed for diverse resistance genes. Examples include the *R* genes *Cf* genes in tomato lines (Hammond-Kosack and Jones 1994), the susceptibility-conferring *R* gene *LOV1* in *Arabidopsis* (Lorang et al. 2007), and the detoxification gene *Hm2* in maize (Chintamanani et al. 2008). Incomplete dominance is generally associated with a gene dosage effect. Higher expression of resistance gene product in homozygous individuals may lead to more effective

perception of pathogen invasion, activation of defensive responses, or elimination of cell damage. The dosage-dependent hypothesis has been tested, to a limited extent, for a few resistance genes. Tomato *Cf* genes (encoding proteins with extracellular leucine rich repeats and transmembrane domain) against leaf mold caused by *Cladosporium fulvum* displayed weakened resistance in heterozygous states (Vidhyasekaran 2007). Homozygous *Cf* lines were capable of responding to a two-fold lower concentration of race-specific elicitors than heterozygous lines (Hammond-Kosack and Jones 1994). In the case of the maize *Hm2* gene (encoding HC-toxin reductase) against the leaf spot and ear mold caused by *Cochliobolus carbonum* race 1, intermediate resistance in heterozygotes has been associated with lower abundance of *Hm2* transcripts (Chintamanani et al. 2008). Although the underlying genes are currently unknown, the resistance phenotypes conferred by *qNLB8.06(Ht2)*_{DK888} as well as other *Ht* major genes are expressed in a similar dosage-dependent manner. This is consistent with the observations that triploid (*Ht1 Ht1 Ht1*) and tetraploid (*Ht1 Ht1 Ht1 Ht1*) maize seedlings displayed a higher level of resistance to NLB than monoploid (*Ht1*) and diploid (*Ht1 Ht1*) seedlings (Dunn and Namm 1970). The dosage-dependent hypothesis and resistance response kinetics can be further characterized by manipulating the isolated resistance gene(s) and its corresponding *S. turcica* effector(s) in follow-up studies.

4. Allele- and genetic background-dependent expression of *qNLB8.06(Ht2)*

The resistance conditioned by *qNLB8.06(Ht2)* varied depending on allele variants and/or genetic backgrounds. The differential performance of *DK888/Pa91* and *S11/NN14B* at *qNLB8.06(Ht2)* in the same genetic background (F₁ hybrid of the Pa91 and the DK888 x S11 NIL) suggested functional allelic diversity. The existence of allelic series for resistance gene(s) at *qNLB8.06(Ht2)* can also be inferred from other

studies. In the multi-parental nested association mapping (NAM) population, differential effectiveness of several parental alleles (relative to the *B73* allele) at *qNLB8.06(Ht2)* were detected in different sub-populations (Poland et al. 2009b). In one of the subpopulations (*B73* x *Oh43*), J. Poland identified *Oh43* as the resistant donor at *qNLB8.06(Ht2)*, suggesting that the *Oh43* allele conferred higher levels of resistance than the *B73* allele. In other reports, the lines *Oh43* and *Oh43Ht2_{NNI4B}* (the *Ht2* isoline in the *Oh43* recurrent background) have been used in qualitative resistance studies (Ceballos and Gracen 1989; Zhang et al. 2007), race identification of *S. turcica* isolates (Dong et al. 2008; Moghaddam and Pataky 1994), and the localization of *Ht2* to bin 8.06 (Zaitlin et al. 1992). In these studies, the *NNI4B* allele showed superior resistance over the *Oh43* allele at *qNLB8.06(Ht2)*. Thus, the *NNI4B* allele was found to be more resistant than *Oh43* allele, and the *Oh43* allele was more resistant than *B73* allele at *qNLB8.06(Ht2)*.

While different alleles at bin 8.06 appeared to contribute varying degrees of resistance to NLB, it remained unclear whether the differential expression was conditioned by a single gene or multiple linked genes. Our observation implied the involvement of at least one linked gene in modulating anthocyanin biosynthesis induced in the incompatible reaction of *qNLB8.06(Ht2)*. Anthocyanins are antioxidants that can protect plant cells against the high levels of oxidative stresses in defense reactions (Hammerschmidt 2005). In the maize – *Cochliobolus heterostrophus* pathosystem, accumulation of anthocyanin has been reported to occur in the uninfected epidermal cells surrounding the lesions (Hipskind, Wood et al. 1996). In our allelism analysis, the accumulation of anthocyanins on diseased tissues was associated with the *Ht2_{NNI4B}* allele and/or the Pa91 genetic background. Genes controlling anthocyanin biosynthesis in maize have been isolated and mapped to several loci on different chromosomes (Bernhardt et al. 1998), including an *a4* locus

(*dihydroflavonol 4-reductase*) residing between *umc2210* and *umc1287* at bin 8.06 (the map location indicated on MaizeGDB). Since the reddish pigmentation was never observed on the resistant plants carrying *qNLB8.06(Ht2)*_{DK888} in the DK888 x S11 background, it is believed that the key resistant gene(s) are apparently not anthocyanin-related. Nevertheless, with certain alleles, the anthocyanin-related gene(s) at bin 8.06 and/or other unlinked loci may contribute additive effects to the resistance of *qNLB8.06(Ht2)*.

5. Map location of *qNLB8.06(Ht2)*

Several major genes and QTL have been isolated by map-based positional cloning. Using this approach, a large number of plant resistance genes have been cloned and characterized [eg. *Pi5-1* and *Pi5-2* against rice blast (Lee et al. 2009), *Rcg1* against anthracnose stalk rot of maize (Broglie et al. 2006), and *Yr36* against wheat rust (Fu et al. 2009)]. In the present study, a total of ~2800 individuals in 39 F₉ or F₁₀ heterogeneous inbred families derived from S11 x DK888 were used to localize *qNLB8.06(Ht2)*_{DK888} from a region of ~19.8 Mb to a region of ~0.46 Mb on the B73 physical map. A high-resolution map surrounding the QTL was constructed using 3 SSR markers and 19 newly developed SNP markers, at a higher density covering the region that is most significantly associated with NLB resistance. Based on QTL analysis employed initially in segregating families and finally with 98 fixed lines carrying various recombinant events in a ~7.4 Mb chromosomal region, *qNLB8.06(Ht2)*_{DK888} was mapped to a 0.46 Mb (1 cM) interval delimited by markers *ctg358-20* and *ctg358-44* at bin 8.06 (143.92-144.38 Mb on the B73 physical map, 10.2-11.2 cM on the S11 x DK888 genetic map). Within the 1 cM interval, *ctg358-20*, *ctg358-5* and *ctg358-37* are closely linked to each other in a 0.08 cM region, whereas *ctg358-44* was located at a distance of 0.92 cM. Although there were 34 out of 98

fixed lines capturing recombination events between *ctg358-37* and *ctg358-44*, we have not succeeded in developing polymorphic markers for this region. *qNLB8.06(Ht2)* can be further delimited by genotyping the 34 lines with more newly-developed markers.

The 0.46 Mb region resides within the intervals of *Ht2* previously estimated from the F₂ populations of A619*Ht2* x W64A (Zaitlin et al. 1992) and W22*Htn1* x A619*Ht2* (Simcox and Bennetzen 1993). It also resides within the map intervals of the NLB QTL identified in the F_{2,3} lines derived from Lo951 x CML202 (Schechert et al. 1999; Welz et al. 1999a), and the NLB QTL identified across the NAM population consisting of RILs derived from 25 diverse maize lines crossed with B73 (Poland et al. 2009b) (J. Poland, pers. comm.). However, some discrepancies were found in previous fine-mapping study using 890 F₂ individuals from the cross of 77*Ht2* and Huobai (Yin et al. 2003). The inconsistent *Ht2* positions as well as the converse order of linked markers observed in the 77*Ht2* x Huobai population suggest that the *qNLB8.06(Ht2)* locus may be divergent among some maize lines.

It has been recognized that the recombination rate for a given resistance locus can vary depending on the similarity of the haplotypes that are paired [eg. the maize *Rp1* locus (Ramakrishna et al. 2002)]. Lower recombination rate flanking the rice *Pi5-1* and *Pi5-2* genes was observed in a population derived from the *RIL260* and *Nipponbare* cultivars, for which the resistant and susceptible alleles from the two cultivars are significantly divergent (Lee et al. 2009). Conversely, R-gene clusters have been widely associated with high recombination frequencies (Bakker et al. 2006; Meyers et al. 2005). In the S11 x DK888 mapping population, the ratio of physical to genetic distance in the ~7.4 Mb region between *umc2199* and *umc1287* at bin 8.06 was ~330 kb/cM. A higher physical to genetic ratio (460 kb/cM) was observed for the 0.46 Mb region of *qNLB8.06(Ht2)*, indicating a lower recombination frequency flanking the resistance gene(s). This implies the possibility of low similarity between

the DK888 and S11 alleles at *qNLB8.06(Ht2)*, and the absence of clustering of homologous resistance genes (which facilitates crossovers) in both alleles. More insights on the evolution of *qNLB8.06(Ht2)* will be gained by detailed investigation of the natural allelic diversity in maize germplasm.

6. Candidate genes underlying *qNLB8.06(Ht2)*

Three compelling candidate genes, including two tandem protein kinase (PK)-like genes and one protein phosphatase (PP)-like gene, were identified within the delimited 0.46 Mb interval of *qNLB8.06_{DK888}*. The two tandem PK-like genes contain the conserved kinase catalytic domain of serine/threonine-specific and tyrosine-specific protein kinases. The PK domain is one of a few conserved domains or motifs shared among R-genes (Xiao et al. 2007). The PP-like gene, on the other hand, has the conserved domain of serine/threonine-specific protein phosphatases, which have been associated with negative regulation of R-gene and non-R-gene mediated defense signaling in rice, *Arabidopsis*, and tobacco (He et al. 2004; Park et al. 2008; Schweighofer et al. 2007). Overall, our preliminary analysis suggested that an R-gene(s) equipped with PK domain and/or a serine/threonine-specific PP gene may underlie *qNLB8.06(Ht2)_{DK888}*. Given the high degree of gene non-colinearity among maize lines (Buckler et al. 2006; Fengler et al. 2007; Fu and Dooner 2002), it is possible that the resistance gene(s) or regulatory sequence(s) does not exist in B73 genotype.

CONCLUSION

Using a HIF-based QTL approach to target a complex genetic region, we identified, characterized and fine-mapped an NLB QTL likely to be identical, allelic,

or closely linked to the known major gene *Ht2*. We provided potentially useful information regarding the resistance spectrum and closely linked markers of the locus. The knowledge will benefit its appropriate deployment in resistance breeding programs. To further delimit *qNLB8.06(Ht2)*_{DK888} and finally isolate the underlying genetic determinant(s), more lines capturing recombination events between flanking markers *ctg358-20* and *ctg358-44* will be screened, and more polymorphic markers will be developed to saturate the interval. Association analysis based on the three identified candidate genes will be tested in a set of ~300 diverse maize lines, which has been evaluated in our group over three years for resistance to NLB (J. Kolkman, pers. comm.). In light of the potential non-homologies between DK888 and B73 alleles at *qNLB8.06*, alternatives to candidate gene analysis, such as chromosome walking or construction of a bacterial artificial chromosome (BAC) library of *qNLB8.06*_{DK888}, may be required. Once the genetic determinant(s) underlying *qNLB8.06* is elucidated, more intriguing hypotheses about the complex genetic architecture, the evolution of resistance gene(s), gene functions and regulations in response to pathogen attack under different environmental conditions can then be addressed.

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CHAPTER 4

CONCLUSION

Numerous studies have documented the co-localization of R-genes⁵, resistance gene analogs (RGAs), defense response (DR) genes, defense response gene homologs (DRHs) and/or quantitative trait loci (QTL) for resistance to different diseases in a range of plant genomes (Chu et al. 2004; Faris et al. 1999; Kanazin et al. 1996; Li et al. 1999; Lopez et al. 2003; Ramalingam et al. 2003; Wang et al. 2007; Wang and Xiao 2002; Williams 2003; Wisser et al. 2006; Wisser et al. 2005). The characteristic distribution of resistance genes/QTL has led to the hypotheses that some chromosomal segments are associated with multiple disease resistance (MDR), and disease QTL may be controlled by specific and/or non-specific genes. Because of the agricultural importance of broad-spectrum resistance and a scientific interest in better understanding it, part of this study focused on characterizing and dissecting genomic regions where multiple resistance genes/QTL co-localized.

In this dissertation, near-isogenic lines (NILs) were generated from three crosses and evaluated for responses to eight maize pathogens with diverse but overlapping lifestyles. The crosses allowed examination of up to six alleles per locus from maize genotypes exhibiting differential degrees of resistance to multiple diseases. Several disease-specific QTL as well as MDR QTL were identified and validated in repeated greenhouse and/or field trials. These included: (1) the B73 allele at bin 1.01-1.02 for resistance to northern leaf blight (NLB), common rust, and Stewart's wilt, (2) the Tx303 and CML52 alleles at bin 1.06 for resistance to NLB and Stewart's wilt, (3) the CML52 allele at bin 6.05 for resistance to NLB and anthracnose stalk rot, and (4) the

⁵ "R-genes" refer to the genes encoding R proteins that are involved in the recognition of specific pathogen effectors (Avr proteins). The R/Avr interaction triggers hypersensitive response and a variety of defense mechanisms.

DK888 allele at bins 8.05-8.06 for NLB resistance. In spite of the varied specificities, all four QTL were localized in MDR hotspot regions (Fig. 4.1). Fine-mapping, gene isolation, and detailed phenotyping will be needed to reveal the genetic basis underlying these QTL, but general aspects regarding the mechanisms, genetic architecture and evolution of these and other MDR-associated loci can be inferred, based on current knowledge of genomic organization, function, and molecular evolution of defense-related genes.

Putative mechanisms underlying loci associated with MDR

MDR phenotype of a chromosomal segment can be attributed to the linkage of multiple genes each with specific effects (mostly R-genes), or the presence of single gene(s) with pleiotropic effects (non R-genes). Depending on the spectra, genes exhibiting pleiotropic effects can be divided into two classes: (1) genes conferring resistance to a wide range of unrelated pathogens (broad-spectrum MDR) and (2) genes conferring resistance to a set of pathogens sharing common features (narrow-spectrum MDR).

A few cases of broad-spectrum MDR have been shown to be associated with key regulatory genes controlling the recognition or signaling of non-host resistance, basal resistance and systemic acquired resistance [eg. *mlo* (Buschges et al. 1997), *npr1* (Cao et al. 1998), and *Lr34* (Krattinger et al. 2009)]. Narrow-spectrum MDR, on the other hand, could result from the recognition of pathogen effectors or features that are common to a more limited range of microbial diversity, or could be associated with genes functioning as downstream components of a variety of mechanisms [eg. pathogenesis-related (PR) genes]. This type of broad-spectrum resistance may be viewed as defense responses targeting certain aspects of plant-microbe interactions. Therefore, only pathogens with similar or partially overlapping modes of pathogenesis

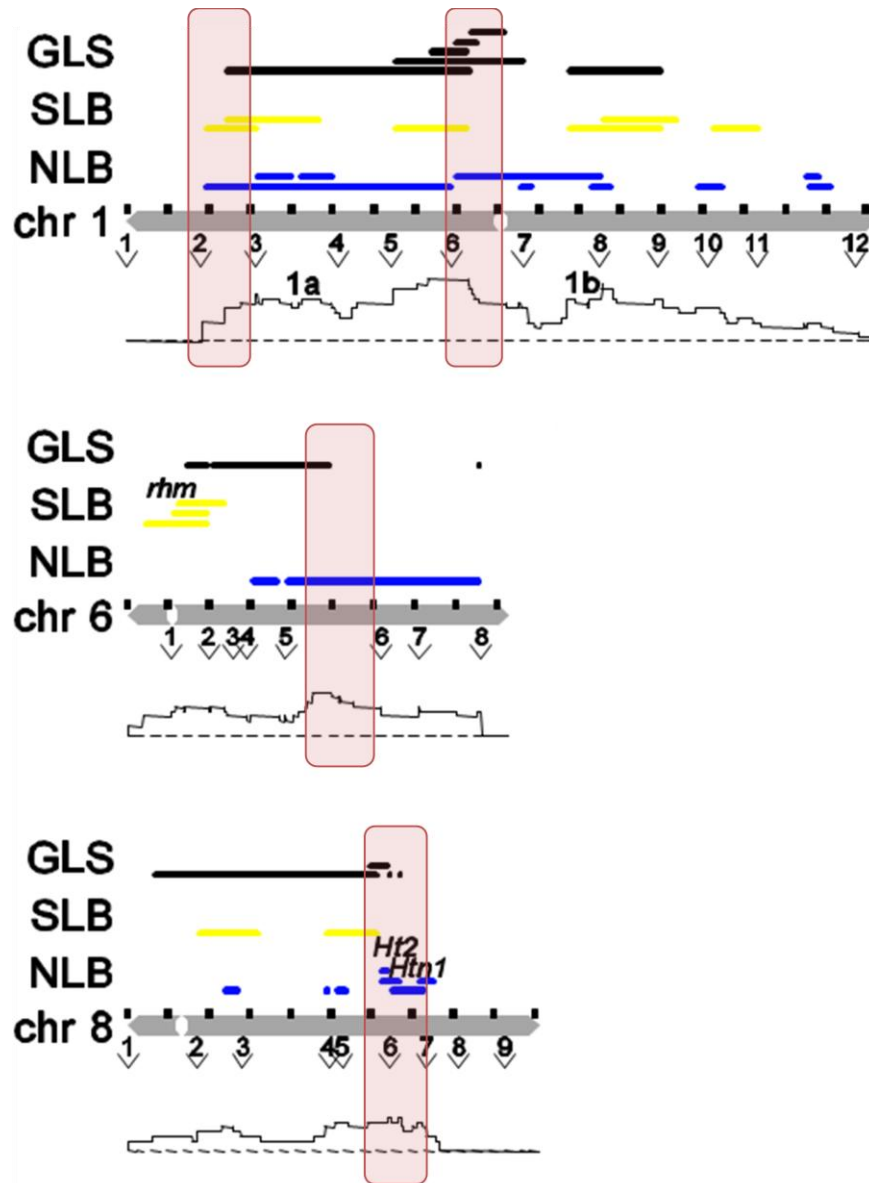


Figure 4.1 Disease QTL at maize bins 1.01-1.02, 1.06, 6.05, and 8.05-8.06 were localized in chromosomal regions associated with multiple disease resistance. The disease QTL consensus map was adapted from the study of Wisser et al. (2006). The maize chromosomes and standard bin positions are shown, with arrowheads and the numbers indicating the start of each given bin. Previously reported QTL for resistance to northern leaf blight (NLB), southern leaf blight (SLB; also known as southern corn leaf blight), gray leaf spot (GLS) are shown as blue, yellow, and black bars above the chromosomes. The histogram below each chromosome represents the frequency of QTL (per cM on the IBM2n map) for resistance to NLB, SLB, GLS, common rust, downy mildew, common smut, ear and stalk rot, *Aspergillus flavus* (aflatoxin), Stewart's wilt, and viral diseases.

would be affected.

Resistance spectra of the QTL at maize bins 1.01-1.02, 1.06, 6.05, and 8.05-8.06 have provided hints for the types of mechanisms that may underlie their MDR patterns. In particular, none of the four QTL were effective against all eight of the pathogens being tested, indicating that the observed resistance is not controlled by genes involving completely broad-spectrum resistance. The DK888 allele(s) at bins 8.05-8.06 were found to confer resistance exclusively to NLB, suggesting that the association of bins 8.05-8.06 with MDR (inferred by clustering of disease QTL) is likely due to linkage rather than pleiotropy. The MDR of the QTL at bins 1.01-1.02, 1.06, and 6.05 suggests that each of the QTL may carry either single pleiotropic gene(s) conditioning narrow-spectrum resistance or multiple specific resistance genes. The low resolution of QTL mapping, however, did not allow the differentiation between pleiotropy and linkage. Because pleiotropic mechanisms that may explain the narrow-spectrum MDR phenotypes of disease QTL have been discussed in detail in Chapter 2, the following part of this section will focus on MDR caused by linkage.

MDR QTL controlled by linkage of genes conditioning resistance to different diseases have been widely recognized. In rye, a 0.06 cM region of chromosome 1 harbors closely-linked genes conferring race-specific resistance to stem rust, leaf rust, and stripe rust diseases (Mago et al. 2005). Two R-genes, *Rx* for resistance to potato virus X and *Gpa2* for resistance to potato cyst nematode, reside in a 0.02 cM region of potato chromosome 12 (Rouppe Van Der Voort et al. 1999). R-gene(s) linked with DR gene(s) may confer MDR. In soybean, an MDR locus (for resistance to powdery mildew and *Phytophthora* stem and root rot) was found to encompass RGAs and novel genes encoding proteins with domains similar to both R-genes and DR genes (Graham et al. 2002). In our study, it was observed that NLB resistance in maize is likely affected by the co-localized *qNLB8.06(Ht2)* and an anthocyanin gene *a4*. Since the

accumulation of anthocyanin in plant cells is associated with reducing oxidative stress, *a4* may involve in defenses in response to other biotic or abiotic stresses.

The physical linkage of R-genes (against same or different diseases) reflects features of genome structure and evolution that have important implications for host defense against diverse and evolving threats. The organization of R genes in clustered families is believed to evolve through local duplications and rearrangements (Hulbert et al. 2001). Frequent unequal recombination and gene conversion occurring in regions carrying clusters of R-genes could lead to fast-evolving resistance genes with chimeric structures and altered functions (Friedman and Baker 2007). Defense-related genes residing in neighboring regions can be regulated by coordinated genetic mechanisms. *Pto*, *Fen* and *Prf* are genes tightly linked on chromosome 5 of tomato. Both *Pto*-mediated resistance to *Pseudomonas syringae* and *Fen*- mediated hypersensitive response to the pesticide fenthion are dependent on the involvement of *Prf* (Salmeron et al. 1996). Analysis of a complex *RPP5* locus in *Arabidopsis*, which consists of multiple R-genes in response to *Hyaloperonospora parasitica* (causing downy mildew) and *P. syringae*, provided evidence that multiple genes in the cluster are coordinately regulated by transcriptional activation and RNA silencing (Yi and Richards 2007). In view of the prevalence of co-localized R-genes and/or DR genes (Chu et al. 2004; Faris et al. 1999; Li et al. 1999; Ramalingam et al. 2003; Wang et al. 2007), it is likely that coordinated regulation of different types of resistance genes may play a key role in broad-spectrum resistance.

Homoeologous relationships among loci associated with MDR

Comparative analyses have identified homoeologous relationships between some of the regions associated with clusters of defense-related genes and/or QTL (Grube et al. 2000; Jo et al. 2008). Resistance specificities of these orthologous chromosomal

segments, however, are not necessarily conserved. Conserved sequences and functions (against closely-related pathogens) of R-genes have been observed at the rust resistance *Rp1* loci of maize and sorghum (McIntyre et al. 2004), and the powdery mildew resistance loci *Mla1* of barley and *TaMla/Pm3* of wheat (Zhou et al. 2001). On the other hand, genome-wide comparative analyses in ryegrass and rice (Jo et al. 2008), as well as in tomato, potato and pepper (Grube et al. 2000), revealed that majority of the homoeologous resistance loci are associated with resistance to unrelated pathogens.

Preliminary examination identified potential macro-colinearity of MDR regions in maize and rice, although with some ambiguity. For the QTL at maize bins 1.01-1.02, 1.06, 6.05, and 8.06, homoeologous MDR regions were found in the rice genome (Fig. 4.2). Part of two rice segments on chromosomes 3 and 10, corresponding to maize QTL at bin 1.01-1.02, were associated with RGAs and QTL for resistance to rice blast and sheath blight. Corresponding to maize QTL at bin 1.06, homoeologous rice segments on chromosomes 8 and 9 were associated with RGAs and QTL for resistance to rice blast, sheath blight and yellow mottle virus. Part of rice segments on chromosomes 1 and 5, corresponding to maize QTL at bins 6.05 and 8.05-8.06, on the other hand, were associated with RGAs and QTL for resistance to rice blast and sheath rot. While some degree of macro-colinearity at the genomic level was observed, it must be acknowledged that these MDR-associated regions are poorly resolved and not fully overlapping. A significant proportion of the homoeologous regions of rice are not associated with any previously identified disease QTL or RGAs. The ambiguity caused by low resolution of QTL mapping is consistent with the results of previous analyses of this type (R. Wisser, pers. comm.). Fine-mapping and eventual gene isolation will allow the evolutionary relationships to be clarified. Meanwhile, the initial maize-rice comparative examination provided some evidence that the four

identified QTL are worth investigating to determine whether they may follow a similar macro-colinearity pattern as many other disease QTL/genes clustering regions.

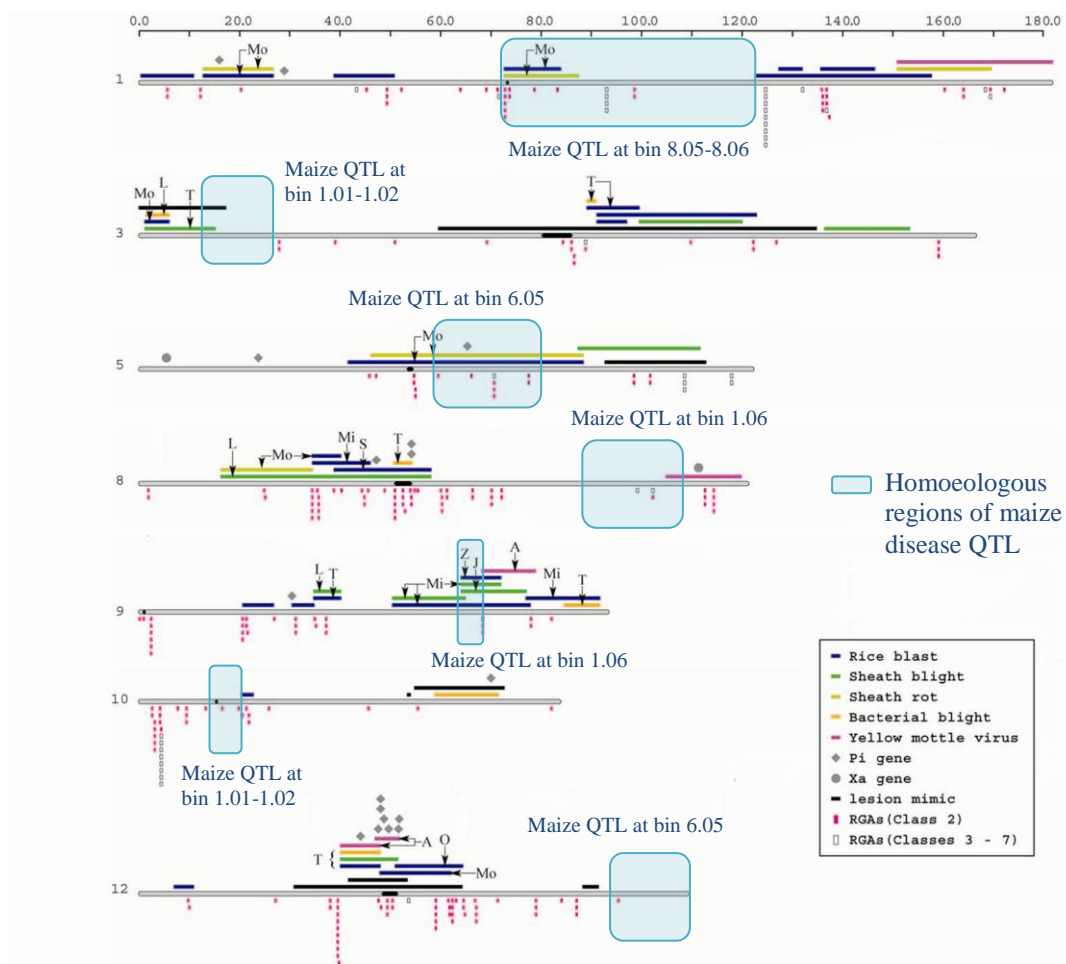


Figure 4.2 Rice homoeologous regions corresponding to maize QTL at bins 1.01-1.02, 1.06, 6.05, and 8.05-8.06. The integrated disease QTL map of rice is adapted from the study of Wisser et al. (2005). Rice chromosomes are shown as gray bars. The maize QTL intervals used for comparative analysis were 16-27.4 Mb for bins 1.01-1.02, 170-190 Mb for bin 1.06, 140-149 Mb for bin 6.05, and 135-155 Mb for bins 8.05-8.06 (all based on the B73 physical map). The maize-rice synteny blocks were determined using SyMap [Synteny Mapping and Analysis Program; <http://www.symapdb.org/projects/poaceae/>; (Soderlund et al. 2006)] and Gramene database [<http://www.gramene.org/> (Jaiswal et al. 2006)]. Preliminary examination showed that all the orthologous rice regions are associated with resistance gene analogs (RGAs) and/or disease QTL.

Putative evolution of genes underlying loci associated with MDR

Different selective forces have been associated with the evolution of different types of genes in plant immune systems. Purifying selection⁶ and directional selection⁷ have been reported to be the dominant forms of selection acting on DR genes (downstream of R-genes). Genes involved in the salicylic acid-, jasmonic acid- and ethylene-dependent signaling pathways in *Arabidopsis* have been found to be mainly under purifying selection (Bakker et al. 2008). Other DR genes, such as defensin gene family members in *Arabidopsis* (Silverstein et al. 2005), and genes encoding chitinase, proteinase inhibitors, peroxidase and oxalate oxidase in diverse plants (Scherer et al. 2005), have been documented as under directional selection. Investigations of nucleotide variation in R-gene loci, on the other hand, revealed evidence for balancing selection or transient/frequency-dependent selection⁸. The maintenance of variants at R-gene loci has been hypothesized to be important in allowing plants to respond to rapidly evolving pathogen effectors (Bakker et al. 2006).

While the evolutionary patterns of R-genes and DR genes have been increasingly uncovered, the evolutionary history of resistance genes within complex MDR loci remains poorly understood. Nevertheless, available lines of evidence support the existence of homoeologous MDR loci in plants. Positional correspondence of QTL/genes for resistance to related/unrelated pathogens suggests conservation of defense-related genes in certain chromosomal regions. The non-conserved disease- and race-specificities of homoeologous MDR loci further implies divergent evolution of individual genes underlying complex resistance loci. That is, since disease

⁶ Purifying selection, also known as negative selection, eliminates or reduces the frequency of alleles carrying deleterious mutations.

⁷ Directional selection, also known as positive selection, increases the frequency of alleles carrying an advantageous mutation.

⁸ Transient/frequency-dependent selection occurs when the fitness of a genotype is dependent on its frequency in the population. This type of selection leads to the maintenance of protein variations over variable periods of time.

pressure can be highly divergent in various geographical areas, local pathogen populations will select for plants that evolve new R-genes able to detect pathogen effectors, or new functional alleles of DR genes able to confer higher levels of resistance. Due to crosstalk between pathogen-triggered defense signaling and other physiological pathways, different environmental factors may also contribute to shaping the evolution of resistance genes. In the hypothesized model, except for the aforementioned purifying, directional, balancing, and frequency-dependent selection that may act heterogeneously on an array of R-genes and DR genes, the evolution of linked genes may be driven by the combination of individual selective forces. As a result, selection may act on a haplotype comprised of multiple genes within a complex locus rather than on a single-gene basis.

Two recent studies provide insights into the functional diversification of linked arrays of genes at homoeologous resistance loci. Compared to the *Fusarium* wilt resistance locus *I2* in tomato, the homoeologous *R3* locus in potato has undergone a significant physical expansion and has evolved to confer late blight resistance (Huang et al. 2005). Sequence analysis suggested that the pepper *Bs2* gene for resistance to *Xanthomonas* wilt is a fractionated ortholog⁹ of members of the *Rx/Gpa* locus in potato (Mazourek et al. 2009). *Rx* and *Gpa* are genes controlling resistance to potato virus X and potato cyst nematode, respectively. These results indicate the complexity of evolutionary mechanisms at resistance loci.

The hypothesized divergent evolution of genes within resistance loci may also explain the observed functional allelic series at MDR QTL in different lines (within species). In barley, a QTL study using an RIL population detected two coincident QTL respectively for resistance to powdery mildew and net type net blotch, with

⁹ Complex orthologous relationships found between the genes within the *Bs2* and the *Rx/Gpa* loci are associated with cycles of gene duplication, deletion and recombination (“fractionation”).

different parental donor lines as beneficial alleles (Yun et al. 2005). In maize, evaluation of the nested association mapping (NAM) population for resistance to NLB, gray leaf spot (GLS), and southern leaf blight (SLB; also known as southern corn leaf blight) led to the identification of 23 QTL affecting resistance to more than one disease. At the MDR loci, alleles exhibiting positively-, negatively-, or non-correlated resistance effects for different diseases were observed (there were alleles conferring resistance/susceptibility to some/all of the three diseases) (J. Poland, pers. comm.). Considering the diverse geographical and breeding origins of the parental lines of the NAM population, it is reasonable to speculate that the differential allelic effects at MDR QTL are the consequence of clusters of genes that coevolved with various pathogen populations.

Implications for resistance breeding

In order to protect plants from diverse pathogens in different environments, identification and utilization of broad-spectrum resistance has been an important goal in resistance breeding programs. While genomic regions associated with MDR have been widely observed, through phenotypic evaluation or compilation of disease resistance studies, certain considerations should be acknowledged. Despite the clear implication of MDR QTL based on meta-analyses of resistance QTL/gene mapping, chromosomal segments that confer MDR were more rare than one might expect. By phenotypically testing selected alleles at MDR-associated regions, it appeared that alleles for disease-specific resistance or narrow-spectrum MDR are more common than those conferring broad-spectrum MDR, which were not found at all. This observation is consistent with MDR QTL mapping using the NAM population, in which multi-parental alleles of QTL were analyzed at high resolution (J. Poland, pers. comm.). In light of the observed allelic diversity at disease QTL, the term “MDR

QTL” may be more clearly defined as “loci conditioning multiple disease responses (rather than resistance)”, as a given allele does not always, or in fact, rarely confer effective resistance to multiple diseases.

Functional variation and complex genetics can be associated with disease QTL, especially those characterized by multiple resistance genes/QTL clusters. Available lines of evidence have implied that durability of resistance is related to non-R/avr gene recognition, non-specificity, and incomplete levels of resistance (details discussed in Chapter 3). For any identified disease-specific or MDR QTL, information about the resistance spectra and underlying mechanisms will not only help predict their long-term performance, but also improve the breeding strategies. For the purpose of developing a crop with MDR characteristics, one could choose to manipulate single or multiple genes with pleiotropic effects, and/or to combine (pyramid) linked/unlinked genes with specific effects in a complementary fashion. In-depth phenotypic characterization and genetic dissection will be key to making these options feasible.

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